

Bringing the market to technology

Too many fields of modern technology contribute less than they might to social well-being because they are regulated nonsensically; telecommunications are the glaring example.

WHOEVER said that the capitalist West lives by the free market in goods and services? Or even by Adam Smith's precept that to let the prices of goods and services reach their own level is to ensure that the most efficient producers of these commodities will become the chief suppliers of them? But now, the "invisible hand" that Smith supposed would guide communities practising internally free trade towards greater contentment seems more often to be a dead hand, especially in the exploitation of technology. Cramped trade is nowhere more notoriously obtrusive than in telecommunications, but international air transport is another black spot. In communications, it is not even possible to pretend that the lives of innocent members of the public will be endangered by the unfettered use of new equipment.

While Washington awaits a sign that President Bill Clinton will make a fight before the end of the year for the new GATT treaty (and that he will win), further evidence of the cramping of technology emerges by the day. Leaders of the new Republican Congress in the United States, for example, have been saying that they will reintroduce a telecommunications bill as soon as possible after the beginning of the new Congress in January, but they do not have it in mind that there will thereafter be a free market in telecommunications.

There is no prospect that the result will be a market economy in telephony. The biggest issue is whether the regional telephone companies created by the breakup of the old Bell System ten years ago should be free to sell long-distance services, partly in compensation for the way their business is being eaten into by cellular telephony. Yet nobody seems to venture the thought that the subsidy of local telephony should be ended. Nor is it on the cards that there will be an end to the practice of the Congress of regulating (through the Federal Communications Commission) the prices charged by cable TV companies.

Pay-TV

Things are no better (and are often worse) in Europe. Only this week, Mr Michael Heseltine, the minister responsible, has decreed that British Telecom, the privatized ex-nationalized industry, will not until 1997 be allowed to sell pay-TV signals through telephone connections for fear of complicating life for the cable-television companies to whom franchises have only recently been let. Yet there is no evidence that the two services would compete head-on, and it makes no sense that the consequence of this arrangement is that there will be two physical networks for broad-band signals

under all of Britain's streets.

On the mainland of Europe, conditions are even worse. The European Commission has decreed that it will be another three years before national telecommunications organizations will be required to accommodate other long-distance telephony companies on their networks (as British Telecom has been obliged to do for the past eight years). And in preparation for what lies ahead, existing national (and still nationalized) communications companies (such as France Télécom and Deutsche Telekom) are making strategic alliances with each other.

Japan

Japan is a different can of worms. The main telecommunications provider, NTT, is being privatized by degrees, but it remains to be seen how liberally the new company will be regulated. British experience shows the virtues of a tough regime that, among other things, prevents cross-subsidies of one service by another. As the technology has turned out, the relative cost of providing long-distance and short-distance connection has changed dramatically in favour of the former. Unless these costs are faithfully reflected in the prices charged, the socially and economically more valuable services will be relatively neglected, to everybody's loss.

Politicians, who usually hold the reins, will unfortunately not see things that way. And the British experience, while encouraging in some respects, should also be a warning to others. The breathtaking pace of innovation in the past decade has taken two forms. The devices used for communications have been enormously diversified while the means by which signals may be transferred from one place to another have similarly multiplied. The Internet may be a pale shadow of Vice-President Al Gore's superhighway, but its most devoted users are mostly ignorant of how the signals cross the world, and often pay nothing for their use of it. And that is only the beginning. How can the regulators cope?

It is in their and everybody else's interest that some ground rules should be widely understood. If there is a capital installation of high cost, such as a national telephone network, it is best that it should be turned into a common carrier, made accessible to all potential users, even mutual competitors. In retrospect, in other words, British Telecom should have been privatized in two parts — a common-carrier network and a telephone company. Then only the carrier would have needed regulation to prevent the abuse of monopoly. But different kinds of carriers need to be separated.



rately owned and managed, so that they can compete effectively against each other. That way, politicians' tasks could be delegated to the technical people (mostly economists) who can compare marginal prices and marginal costs. That, and not fixing the price of cable television subscriptions, is what governments should be aiming at. □

No CBT for NPT

Hopes that there would be a test-ban deal before the NPT must be renewed are fading fast.

THE twenty-fifth anniversary of the Nuclear Non-Proliferation Treaty (NPT) falls next year. In just six months, there will be a conference at Geneva at which the present parties to the treaty, getting on for 150, will have to agree (or otherwise) to the continuation of the treaty. That fateful occasion appears to have stirred up very little dust in the big wide world. To be sure, the United States has brokered what may be a useful deal with North Korea, but the state of nuclear weaponry in the former Soviet Union remains confused and a threat to peace. The problem for the nuclear powers, most of whom want to prevent the spread of nuclear weapons, is that they will need to win continuation of the NPT with tangible proofs that they have wound down the arms race of the Cold War. A Comprehensive Test-Ban Treaty would by itself have been sufficient.

Now, alas, the prospects of such an agreement between the existing nuclear powers are poor. The negotiating teams at Geneva have just awarded themselves a long recess, and can hardly be expected to secure this prize in the few weeks they will have in which to tinker with the treaty before the NPT conference begins. By some accounts, France has made the most clamant objections to a deal, but Britain has also been insisting on the need for tests of the Trident warheads now being built. China, a participant at the talks, has by contrast been comparatively silent on the subject, perhaps counting on France and Britain to keep the argument for continued testing bubbling away. It is all a bad business, which needs to be tackled with more urgency than any government (the American included) appears to sense. □

Big Bang gone quiet

Cosmologists had better say something about the recent measurement of the Hubble constant.

LIKE the Sherlock Holmes dog that failed to bark, cosmologists have been remarkably silent about the two measurements of the Hubble constant reported in the past few weeks (M. J. Pierce *et al. Nature* **371**, 385–389; 1994 & W. L. Freedman *et al. Nature* **371**, 757–762; 1994). The essence of the case, it will be recalled, is that it has been possible to identify stars that resemble Cepheid variables in other galaxies of the Virgo galaxy-cluster and, using them as better standard candles than any previously available, to estimate the scale

of the apparent speed of the expansion of the Universe. That is Hubble's constant, which, at about 80 km per second per megaparsec, is near the high end of range in which previous estimates of this quantity have clustered.

Remarkably, the first of these measurements was carried out at the Canada–France–Hawaii Telescope in Hawaii. Although the age of the Universe inferred from this figure must depend on other assumptions there seems little doubt that the inferred age of the Universe is nearer 10 billion years than twice as much, and that the Universe is therefore 'younger' than some of the stars in the globular clusters of our own Galaxy, which appear to be older than some 14 billion years.

On the face of things, then, the new measurements are a blow for the Big Bang account of the beginning of the Universe, although not necessarily a fatal one. It could, for example, be the case that the motions of the two Virgo galaxies in which the apparent Cepheids have been found are so inaccurately known that their use as standard candles is misleading. Or it may be that what appear to be Cepheids in the two other galaxies nearer the centre of the Virgo cluster are somehow systematically different from those used in the past half century as the most reliable indicators of distance in the local galaxy. Or maybe the standard model of the expansion of the Universe needs correction. Any or all of those explanations would be credible, and even creditable. But a continued silence will only give the world the impressions that the cosmology of the past 30 years is under a cloud. □

Regressive taxation

The British government has hit on a way of making taxation popular and seeming to be fun.

THE British have been in a fever of literal speculation for the past two weeks, handing over pound (sterling) coins for a chance to participate in the brand-new national lottery, launched on 13 November by the prime minister, Mr John Major. Evidently, he was not for nothing Chancellor of the Exchequer for a brief spell; the lottery organizers collected £37 million in the first week of operation. Roughly half of that will go to the government, and a quarter to what are called good causes, notably the arts, various aid organizations and a fund with which to mark the coming millennium.

As a gamble, the British lottery is therefore not a good buy. Casinos with roulette wheels offer a much better chance of winning back, and more, what gamblers call their 'investment'. So, oddly enough, do slot-machines. The organizers are a company called Camelot. They say they will photograph all the winners of prizes exceeding £10,000 to make sure that those concerned are not laundering tainted money.

In reality, the most eager participants in the lottery will be those most in need of riches, mostly the poor. Occasionally, of course, they will win but mostly half of their investments will go straight to the Treasury. Voluntary taxation would be the proper name for what is planned. Only a clever government could make regressive taxation popular. □

Mouse gene repository plans likely to persuade Italy to stay in EMBL

Munich. Italy is almost certain to withdraw its notice to quit the European Molecular Biology Laboratory (EMBL), following last week's approval by the EMBL council of a plan for a European-level mouse gene repository and research facility near Rome.

The plan would include an application to the European Commission in Brussels for joint funding for the repository. The Italian government, which had planned to leave EMBL at the end of this year, is now expected to decide within the next few weeks to remain a member.

Last December, Italy gave 12 months notice to leave EMBL, claiming that it was not getting value for money on its subscription. It pointed out, for example, that although it pays DM10 million (US\$6.4 million) to EMBL, 16 per cent of the EMBL budget, there are relatively few Italian staff at the main laboratory in Heidelberg (see *Nature* 366, 604; 1993).

Fotis Kafatos, the new director-general of the laboratory, put forward two proposals in February for increasing the value of EMBL to southern European countries. One was to recruit more staff from such regions, the second to set up EMBL regional research groups at host institutes in southern Europe.

The measures were welcomed by many Italian biologists. But the Italian government felt they were insufficient, and wanted EMBL to establish an outstation in Italy along the lines of those in Germany (at the DESY accelerator in Hamburg), France (at the Institut Laue-Langevin in Grenoble) and the United Kingdom (the European Bioinformatics Institute, which is situated

near Cambridge).

After reassurances at its meeting last week that Italy would be unlikely to leave the organization if a compromise could be reached, the EMBL council voted unanimously for a scheme that combines elements of both proposals. This would involve supporting a bid from several national research organizations to create a European-level repository for mouse genes and establishing four EMBL research groups at laboratories donated by the Italian chemical company Enichem at Montorotondo, near Rome (see *Nature* 369, 699; 1994).

The research groups at the centre would replace the four EMBL regional groups that were planned to be spread throughout Italy. The Consiglio Nazionale della Ricerche (CNR), Italy's basic research organization, will also establish research groups to work alongside the EMBL scientists.

The proposal to establish a repository for mouse genes at the site is being put forward by the major research organizations in the four countries in which EMBL has laboratories. Earlier this month, these four research organizations, Germany's Max Planck Society, the UK's Medical Research Council (MRC), Italy's CNR and France's Centre National de la Recherche Scientifique, set up a committee of scientists to assess the suitability of the Montorotondo facilities for this purpose.

Peter Gruss from the Max Planck Institute for Biophysical Chemistry in Göttingen, who co-chairs this committee with Peter Rigby from the MRC's National Institute for Medical Research, says that if the com-

mittee approves the site — there are no other candidates at present — the consortium of research organizations will submit a formal proposal to the commission for funds for genetic repositories in Europe available under the Framework IV programme. The commission expects to put out a call for proposals in the next few weeks.

They will not know if they have been successful until at least next spring. But Gruss is optimistic of its chances, as the idea has been discussed extensively within the commission's research directorate, and has the active support of Antonio Ruberti, the research commissioner. Gruss adds that he sees no reason to suppose that Ruberti's successor, Edith Cresson, would be less supportive.

Kafatos says that he will be "willing to assist in any way to get [the proposal] approved", but stresses that it is independent of EMBL, and not directly part of the deal to draw Italy back into the EMBL fold.

If all goes according to plan, work could start at Montorotondo by the end of next year.

Allison Abbott

Balzan prize shared by astrophysicists and neurobiologist

Rome. Two astrophysicists, Sir Fred Hoyle and Martin Schwarzschild, and the French neurobiologist René Couteaux, are this year's recipients of the Balzan prize for lifelong contributions to science. The prize, worth SFr350,000 (US\$266,000) to each recipient, is awarded to natural and social scientists in areas not covered by the Nobel prize.

Hoyle, who is 79, and Schwarzschild, 82, were recognized for their work on stellar evolution. Hoyle demonstrated that carbon and heavier elements can be produced by nuclear reactions in stars. He also predicted — correctly — that the carbon nucleus could exist in an excited state with just the right energy to allow its formation from three helium nuclei.

Working with the German-born Schwarzschild, Hoyle developed the theory that low-mass stars can evolve into very luminous giants, an important stage in understanding the physical processes of stellar evolution. Schwarzschild, who has spent most of his career in the United States, also pioneered the use of telescopes carried by balloons for taking images of the Sun, planets and stellar systems.

Couteaux, who is 85, described during his half-century of active research the detailed structure of the neuromuscular junction. **A. A.**

Faraday successor targets the brain

London. **Susan Greenfield (right),** a lecturer in pharmacology at the University of Oxford, will become the first woman to present the Royal Institution (RI) Christmas Lectures later this year when she gives five lectures for children from the age of 10 upwards on the workings of the brain.

Among the subjects to be covered in the 165th series of the lectures, started by Michael Faraday in 1826 and now broadcast annually by the BBC, are methods for studying the brain, how it receives signals about its environment and converts these into electrical impulses and chemical transmitters, the brain's development, memories and intelligence, and what makes a human being unique.

Greenfield is seen with a statue of Faraday, one of the RI's most prominent scientific members — and a hero of the former prime minister, Lady (Margaret) Thatcher. □

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European research heads move to sharpen their talons

Munich. The heads of Europe's main national research funding organizations, who have been meeting informally for nearly two years to discuss how their organizations can work more closely with the European Commission's (EC) research programmes, have decided to set themselves up as a formal body to give themselves a stronger voice within the European Union (EU).

Meeting in Madrid last week, the group, known as the Eurohorcs (for European heads of research councils) decided to elect a president when it meets next spring. In the meantime, José Mato of Spain's CSIC is temporarily holding the reins of a newly formed steering committee.

The impetus for the formal establishment of the group was its members' disappointment that the EC research commissioner, Antonio Ruberti, excluded a Eurohorcs representative when he set up his 100-strong advisory European Science and Technology Assembly (ESTA) last September (although many of those appointed to ESTA also happen to be Eurohorcs).

Such a representative would have had a much more powerful voice in ESTA because he or she could have spoken for all EU research organizations, says Jan Borgman, head of the Dutch NWO, who is also chairman of the assembly.

Borgman says that national research councils have a strong interest in EU research programmes, which have much larger budgets now than in the recent past.

The Eurohorcs want a greater influence on EU research policy and to be more closely involved in coordinating both national and European-wide research programmes. They also want more direct links with the EC in Brussels.

Such plans could conflict with the goals of the European Science Foundation (ESF), based in Strasbourg, which has also been hoping to develop a similar advisory role in Brussels (see *Nature* 366, 193; 1993).

Many smaller EU countries would have liked the Eurohorcs and the ESF to have joined forces, combining the resources of the ESF's secretariat and scientific staff with the politically powerful Eurohorcs in order to achieve shared aims. But some larger countries preferred a more direct role in Brussels, and did not want their influence diluted through ESF.

Both sides insist that there is no conflict of aims, and representatives from each will attend the other's meetings. Meanwhile the ESF is continuing to carry out a strategic reappraisal of its activities and a search for a new identity. Both will be top of the agenda when its general assembly meets in Strasbourg this week.

Allison Abbott

NIH seeks bids for vector centres for gene therapy

Washington. The US National Institutes of Health (NIH), concerned that clinical trials of gene therapy are being held back because of the difficulty faced by researchers in obtaining suitable vectors, have announced that they will award \$3.5 million next year to establish a small number of National Vector Laboratories.

In requesting applications to run these centres, the NIH says that the failure to accommodate researchers' needs for vector production "constitutes a barrier to progress in the field of gene therapy".

Several factors have given rise to the present situation. First, producing new vectors is expensive, as those to be used in humans must be manufactured in facilities that meet stringent requirements laid down by the Food and Drug Administration (FDA).

Second, many of the small companies that might have produced vectors on a contracts basis have recently gone out of business. Finally, biotechnology companies still in a position to do so face a shortage of capital, and are under pressure from investors to produce immediate results.

Gary Nabel, an investigator for the Howard Hughes Medical Institute at the University of Michigan in Ann Arbor, describes the NIH initiative as "less than would be ideal, but we have to start somewhere". Nabel's group is one of the few that make their own vectors and may bid to become a national centre.

Researchers at Michigan are already discussing possible collaborations with some of the other groups in the United States producing vectors about ways of

making them available to investigators.

Malcolm Brenner, who is developing gene therapies at St Jude's Hospital in Memphis, Tennessee, describes vectors as "the key to gene therapy". Vectors include various types of virus whose genetic material is manipulated so that they cannot reproduce some types of lipid compounds. The vector targets specific cells to incorporate new genetic material into the genome. Nearly all aspects of their mechanism need to be improved. "Vector development is primitive," says Brenner. "We only have the model-T Ford."

According to Brenner, the NIH's decision to fund between one and three National Vector Laboratories means that collaboration between academic institutions and industry may be simplified. At present, investigators often need to negotiate with three or four companies for materials when establishing gene-therapy protocols. Each company is concerned that it may gain nothing from the collaboration while enhancing its competitors' products, and negotiations can therefore be protracted and complex.

The aim of such national laboratories will be to produce clinical-grade vectors for human gene therapy. The NIH expects that a few grants will be awarded before next August and that funding will continue for five years.

The resulting vectors will already have had preclinical testing, and will be made available to investigators with protocols approved both by the FDA and, if necessary, the NIH's Recombinant DNA Advisory Committee.

Helen Gavaghan

New element spurs naming protest

London. A controversy over the naming of new elements has been aggravated by the announcement last week that researchers at the Gesellschaft für Schwerionenforschung (GSI) in Darmstadt, Germany, have successfully created element 110 — but are refusing to propose a name for it.

Many physical chemists have been upset about the announcement by the International Union of Pure and Applied Chemistry (IUPAC) in September that it plans to name element 106 rutherfordium, after the New Zealand physicist Ernest Rutherford, rather than seaborgium. Seaborgium was proposed by the discoverers of element 106 at the Lawrence Berkeley Laboratory (LBL) in California in honour of the Nobel prizewinner Glenn T. Seaborg (see *Nature* 371, 639; 1994).

Although the paper describing the new

element is not expected to be published until the end of December (in the journal *Zeitschrift für Physik*), the GSI group, led by Sigurd Hofman, were sufficiently confident of their 'discovery' to announce it last week. The laboratory had earlier created elements 107, 108 and 109, and, according to Peter Armbruster, head of nuclear chemistry at GSI, the group is more confident of its latest discovery than when it announced '109' in 1984.

But the discoverers are still unhappy with the names proposed for the earlier elements by the IUPAC Commission on Nomenclature of Inorganic Chemistry. GSI had suggested nielsbohrium (107), after physicist Niels Bohr, hassium (108) after Hassia, the latin name for the region in which GSI is located and meitnerium (109) after the physicist Lise Meitner. But the names proposed by the IUPAC▶

Gloomy prospects face biodiversity treaty

Washington. As delegates prepare to meet next week in the Bahamas to negotiate details of the international Convention on Biological Diversity, signatories to the treaty, approved by the Earth Summit in Rio de Janeiro in 1992, remain as divided as ever on several important issues.

These range from how the convention should be funded to the possible implications of a bid to impose a moratorium on trade in genetically engineered plants and animals. Furthermore, with the United States still putting off ratifying the treaty, the prospects for settling those issues appear worse than at any time since the convention came into being.

President Bill Clinton signed the biodiversity treaty last year. But the Senate failed to ratify it, leaving the United States as merely a non-voting 'observer' at the Bahamas meeting. So far, 167 countries have signed the convention and 97 have ratified it, including all the other major industrial nations.

Much of the business at this first conference of parties, which runs from 28 November to 9 December, will be administrative, setting up rules and procedures. But the signatories will also face some of the divisive issues that have so far been avoided through vague wording of the treaty — such as the transfer of technology to developing nations, and the sharing of patent royalties for products developed from native biological resources.

The agreed interim funding mechanism is the Global Environment Facility (GEF), administered by the World Bank. The United States and other industrial nations would like to make that arrangement permanent. But many developing nations, who want a greater say in which projects receive fund-

ing, are likely to resist.

Intergovernmental meetings held over the past two years to lay the groundwork for next week's conference have made little progress in deciding what kinds of projects should even be funded. They have agreed on easy projects, such as taking biological invento-

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Still at issue: how to fund projects aimed at preserving genetic resources.

ries. But they have argued over how and where conservation programmes should be set up — for example, whether money should be spent preserving known 'hot spots' of biodiversity, or be more evenly distributed between countries.

Another potentially explosive issue is 'bio-safety' — the purported danger of introducing genetically engineered organisms into the natural environment. Even though the matter is not on the agenda, several nations including India, as well as a number of environmental groups, want to raise it.

Richard Godown, a senior vice-president at the US Biotechnology Industry Organization, which represents the interests of biotechnology companies, says that he does not believe a moratorium on trade in genetically engineered plants and animals

will be agreed. But he describes it as a "dangerous idea to have floating around" at the Bahamas meeting.

Concern about such potential restrictions has fuelled US opposition to the biodiversity convention. Conservative Republicans in particular cite the treaty's vagueness on protecting intellectual property rights, and possible — though some say unlikely — restrictions on private property owners, as reasons not to sign.

Following the recent Republican congressional victories, the committee that oversees ratification of the treaty will now be chaired by one of its most vigorous opponents, Senator Jesse Helms of North Carolina. Republican opponents such as Helms are gambling that, even if the United States does not participate as a formal voting member, it will still wield influence at next week's meeting through its position as the largest donor to the GEF. As

a Republican policy paper written in September says, "if the United States has so little support from its friends that no one will assist [it] by objecting to consensus on issues adverse to our interests, then the [Clinton] Administration had no business signing this convention".

But US reluctance to ratify may also encourage developing nations to pursue their own agendas at the meeting. Even if the United States dropped out of the convention, the GEF would still amount to some \$1.5 billion, nearly half of which would be used for programmes under the biodiversity convention.

Many US conservationists and biologists say the United States will be the loser if it chooses not to ratify. "The United States lost an amazing amount by not signing the convention early," says Daniel Janzen of the University of Pennsylvania. Now, he claims, US researchers are viewed with suspicion when they go prospecting for biological resources in other countries.

Godown agrees, and warns that if the United States does not sign, developing countries may choose to work instead with biotechnology companies from countries that have agreed to accept its rules.

An indication of the tensions likely to surface at next week's meeting have already come from the Convention on International Trade in Endangered Species (CITES) that ended last week in Florida, and which saw fireworks over, among other issues, proposed new criteria for listing species as protected.

The US delegation successfully fended off an attempt to apply rigid — and scientifically unjustifiable — numerical criteria on population size and size of habitat that would have allowed trade in certain whales, apes, and other species.

Tony Reichhardt

► committee and due to be ratified by the IUPAC Council at its meeting in Guildford, England, next year, are bohrium, hahnium and meitnerium respectively.

"The discoverers have the right to name an element, and IUPAC can accept it or not," says Armbruster. "But if they don't, they should tell you and allow you to come up with another name, not invent another name themselves."

The group are particularly unhappy because they wanted to honour Lise Meitner, who worked with Otto Hahn. He was awarded the Nobel prize for chemistry in 1944 for their joint work on the discovery of the fission of heavy nuclei. But Meitner received nothing and her contribution to the work is generally overlooked. The group did not want the names to be used for adjacent elements.

Their dissatisfaction is echoed by Albert Ghiorso, senior nuclear scientist

emeritus at LBL, and leader of the team that discovered element 106. "The right of discoverers to name elements is sacrosanct," says Ghiorso.

In view of the current dispute, the German group has decided not to propose a name for the new element. It says that it has sent a letter to IUPAC explaining its disagreement with the way the earlier elements were named and that it will "do everything" it can to force IUPAC to reconsider its proposals before the ratification meeting in Guildford.

Ghiorso is confident that, faced with widespread opposition, the IUPAC will back down. And he, for one, will continue to call the elements by the names suggested by their discoverers: rutherfordium (104), hahnium (105), seaborgium (106), nielsbohrium (107), hassium (108) and meitnerium (109).

Maggie Verrall

Hopes and doubts greet Paris AIDS 'summit'

Paris. Leading politicians from 42 nations will meet for an 'AIDS summit' in Paris next week. One outcome will be a declaration committing the signatories to promote prevention — including removal of economic, cultural and other obstacles — and to adopt national policies to help people who are seropositive and protect their rights.

The declaration will also call for international actions in seven areas, including blood safety, cooperation between public and private sectors in research and development, and social and health-care programmes.

Bernard Debré, chairman of the summit organization committee, and the newly appointed French minister of cooperation, has called the meeting a political "first" and "unusual" in that it will produce "practical action". Debré admits that consensus on areas such as sex education is unattainable, but has described any agreement as "already something".

But many AIDS researchers and activists are concerned that the declaration — which they describe as a "list of good intentions" — may prove both unworkable and ineffective. They claim that the difficulty in getting consensus among 42 countries has made it "lowest common denominator" material. Fewer but firmer commitments would have been preferable, they say, for example to increase funding for AIDS programmes and end discriminatory laws.

Activists argue that the declaration's wording is so ambiguous that even Russia, whose parliament last week approved compulsory HIV screening of foreigners, has few qualms about signing it. Critics also argue that Roman Catholic states could avoid promoting condoms, because a provision calling for "promotion of access to means of prevention", includes the caveat that this should be "culturally acceptable".

Act-Up, one of several activist groups represented on the organizing committee,

now describes the meeting as a foregone "failure" and a "step backwards". A member of another group, ARCAT-SIDA, complains that "boosting France's international influence" is now the summit's "sole objective".

Indeed, the meeting has been controversial ever since it was proposed last year by Simone Veil, the French health minister. Many countries opposed it at first, arguing that with various United Nations (UN) agencies combining their AIDS activities into a single programme coordinated by the World Health Organization (WHO), it risked duplicating efforts elsewhere.

Such concern faded in May, when WHO became a coorganizer of the summit and it was agreed to channel any new funds or priorities to emerge into the UN programme. Support from Edouard Balladur, the French prime minister of whom Veil is a political ally, also helped to persuade a meeting of health ministers from the 42 countries — 19 from the developed world and 23 from developing nations — in June to back the meeting.

Despite such misgivings, most agree that the summit will boost flagging momentum for AIDS prevention; industrialized countries have reduced such funding over the past few years. Michael Merson, director of WHO's Global Programme on AIDS, says overall funding from industrial countries is "insufficient", while WHO itself has "not raised nearly the resources it needs to".

Merson denies that the meeting is a "pledging" event. But France seems to have other ideas. Debré has proposed organizing a global AIDS 'téléthon' to raise funds. A similar event held in France recently raised FF300 million (US\$56 million). Moreover, while France is one of the smallest contributors to UN AIDS programmes among the industrialized countries, it has promised FF100 million to support the outcome of the summit and unblocked an extra FF300

million for AIDS activities within bilateral cooperation programmes.

Although France's bilateral efforts are substantial, critics point out that these suffer from a lack of coordination as they fall within the remit of the ministries of foreign affairs and of cooperation, not the ministry of health. Critics add that such funds are often distributed — as in most countries — on political rather than health-care criteria.

Other countries have remained remarkably silent about whether they intend to commit funds. Moreover, observers are concerned that such pledges may not be new money but simply money diverted from bilateral programmes.

Who will attend is another big unknown. Boutros Boutros Ghali, the UN secretary general, Jacques Delors, outgoing president of the European Commission and Helmut Kohl, the German chancellor are expected. But the United States will send Donna Shalala, secretary of state for health, rather than vice-president Al Gore (Shalala is expected to also visit the Institut Pasteur to bury the hatchet in the Gallo/Montagnier affair). Two weeks from the meeting, many invitations remain unanswered.

Merson hopes that the meeting will prove the sceptics wrong. "If we can present convincing initiatives to donor countries, we will get increased resources." The highest priority, he says, is to get better international cooperation between public authorities and private companies to promote research; through a coordinated approach to subsidizing corporate research, sponsoring clinical trials, and making any drug developed available to developing countries.

Research priorities, he says, are vaccines and vaginal microbicides. The latter could revolutionize efforts to prevent heterosexual transmission of HIV, he says, by protecting women, especially in developing countries, who cannot ensure that their partners use condoms (see *Nature* 366, 293; 1993).

WHO is also likely to use the meeting to publicize its recent authorization for the testing of HIV vaccines in developing countries (see page 313). The costs of the trials — estimated at tens of millions of dollars — will probably be met by Sweden and Japan, according to observers, pointing out that Hiroshi Nakajima, the director-general of WHO, is himself Japanese.

Another priority is to improve blood safety. In 1989, WHO tried to set up a Global Blood Safety Initiative, but this foundered because of lack of funding and inter-institutional tensions. After much internal political wrangling, WHO this month set up a planned "blood safety unit" (see *Nature* 369, 429 1994). Any new funds for blood safety coming from the summit are likely to go to this new unit, says Merson.

Declan Butler

Call for international AIDS policy

Paris. Even critics of next month's meeting on AIDS in Paris (see above) accept that it may achieve one positive result: exposing the inability of existing international structures to respond adequately to the AIDS epidemic. "If 42 heads of state won't accept their responsibilities, it simply reveals there is no international policy on AIDS," says a member of the group Act-Up.

There is a growing sentiment, both within such groups and in parts of the AIDS research community, that while the delays and bureaucracy inherent in international politics may be acceptable in economic and other areas, they are unacceptable faced with the urgency of the AIDS epidemic.

Earlier this year, six United Nations

organizations — the UN Children's Fund (UNICEF), the UN Development Programme (UNDP), the UN Educational, Scientific and Cultural Organization (UNESCO), the UN Population Fund (UNFPA), the World Bank, and the World Health Organization (WHO) — combined their AIDS activities into a single "UN Programme on HIV/AIDS".

The programme's aim is to reduce bureaucracy, competing mandates and duplication, and to improve the coordination of fund-raising and programmes. But while the programme's director will be nominated next month, the programme itself will not start until 1996. Between now and then, around 8 million more people will have become infected with HIV.

D. B.

Greater openness urged over nuclear waste storage plans

London. Britain's Royal Society has urged the company drawing up plans for the country's first depository for radioactive waste to include provisions for storing high-level waste — even though the company itself, UK Nirex Ltd, fears that any such proposal could inflame public opposition.

It has also urged greater openness in the distribution of the company's scientific reports, saying that it was "forcibly struck" by the way some of these had been protected from public scrutiny.

The recommendations come in a report published last week by a six-member committee set up by the society at Nirex's request. The group was chaired by Sir Alan Muir Wood, a prominent consultant engineer, to study the scientific aspects of the company's plans for a nuclear waste depository close to the Sellafield reprocessing plant in Cumbria. Nirex's plans to store low-level waste and intermediate-level waste some 650 metres underground have caused a storm of protest, in particular because of concern that water contaminated with radionuclides could rise to the surface (see *Nature* 369, 698; 1994).

The report says that the Sellafield area is hydrogeologically complex, with most groundwater flow occurring through fractured rocks. The task of assessing where water from the potential repository zone (PRZ) flows now, and in the long-term future, is the key to predicting how the sealed repository would perform. "If one had simply a set of geological factors to choose from, it is unlikely that Sellafield would be the first choice," Wood said last week. But, he added, "it doesn't have to be the best site, it has to fulfil certain criteria".

The study group's report also concludes that the identification of the PRZ "appears to have been somewhat premature", and that it should be extended deeper and to the west where conditions appear more stable.

The report points out that "currently available preliminary information indicates that the high salinity of the groundwater and longer flow paths may result in lower levels of radioactivity moving towards the ground's surface". If this were the case, such a depository could also be used for high-level waste. At present, Nirex has no responsibility for disposing of such waste; but the society group points out that there is not even a research programme in the United Kingdom for its disposal.

While praising the quality of much of the work undertaken and commissioned by Nirex, the group says it felt that the "innovative and complex nature" of the work needed to understand the structure of the area, and the need for peer review, meant that the

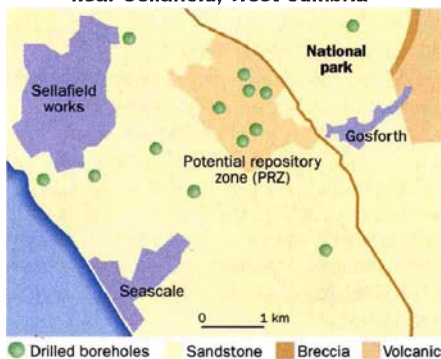
Nirex timetable was too optimistic. Under this timetable, the first deposits of waste would take place in 2010.

Wood also advised Nirex to be as "open as possible" in discussing and publishing its scientific data, enabling wide peer review and the selection of the best site by a "highly iterative interaction" between the scientists and the design engineers.

But the Royal Society backed Nirex's plans for a rock characterization facility (RCF), and said that such a 'rock laboratory' should be built "as soon as is practicable", provided sufficient information was obtained before the hydrogeological regime of the area is disturbed by the RCF.

Nirex applied to Cumbria County Council for planning permission for the RCF in July, and council representatives immediately wrote to the Secretary of State for the Environment asking for the application to be

Planned site of UK nuclear waste depository near Sellafield, West Cumbria



'called-in' for a public inquiry. The council is concerned about the way that Sellafield was chosen from an original list of candidate sites and the lack of sufficient information on findings so far.

In a separate report, the Royal Society calls on the government to develop national policies on both energy generation and the civilian use of plutonium. Its comments come in a response to a consultation document from the DoE on radioactive waste management, which describes issues raised by existing and future waste as "a problem that faces all citizens, whatever their views about the desirability of nuclear power".

Sir Francis Graham-Smith, physical secretary and vice-president of the Royal Society, called on the government to reconsider the possibilities for disposing radioactive waste in the ocean, even though this is at present ruled out by international law. "There are obvious attractions of ocean disposal, and we should keep looking at the science of that," said Graham-Smith. **Maggie Verrall**

Row over new job losses at German microscope factory

Munich. Carl Zeiss, the German company that supplies about a quarter of the world's microscope market, has been told by the local government in Jena, where its east German factory is based, that plans to cut 3,000 jobs in 1996 violate a previous agreement not to make further cutbacks.

The proposed cuts are to be spread throughout the company. But Jena would lose 600 of its 2,000 jobs in the plan, a much higher percentage than the company's other locations. Union officials fear that even more cuts may be on the way, and that the Jena branch may eventually be closed.

Zeiss was founded in Jena in the late nineteenth century. But at the end of the Second World War, when Germany was occupied by the Allied Forces, US troops led a core group of scientists out of eastern Germany to start a new Zeiss company in Oberkochen, Baden-Württemberg.

After reunification, Zeiss Oberkochen merged with the fine optics and mechanics sections of its Jena predecessor and competitor. Zeiss now owns 51 per cent of the company and the remaining 49 per cent is owned by the *Land* of Thüringen, where Jena is located.

But the merger precipitated a financial crisis, prompting Zeiss to reduce its workforce to 16,000, 85 per cent of whom work in Germany. The recent announcement of additional job losses has provoked strikes in both Oberkochen and Jena.

In January, Thüringen, where Zeiss is an important employer, signed an agreement with Zeiss for staff cuts of 800 by 1996. Hans Kaiser, a spokesman for Thüringen, claims that this agreement meant that no additional cuts would be enforced.

But Berndt Räbel, a Zeiss spokesman, says the agreement allowed for additional cutbacks if the company encountered special financial problems. "Jena will remain an integral and vital part of Zeiss," he says, adding that research and development will continue at Oberkochen and Jena.

In an attempt to improve the strained relationship between Zeiss management and the east Germans, the company is reorganizing its four-member board. Peter Grassmann, now a board member of Siemens and widely recognized as an effective negotiator, has been appointed to the board and will also join Zeiss management in Jena.

Kaiser believes that Grassmann's more direct style will help to diffuse tensions and give a fresh impetus to negotiations. Roland Hamm of IG Metal, the trade union that represents Zeiss's employees, says that the appointment of a new board representative can be seen as a first conciliatory step by the management. **Toni Feder**

European patent directive in critical test over genes

London. Six years of effort by the European Commission (EC) in Brussels to harmonize legislation on biotechnology patents in the member states of the European Union (EU) are in the balance. At issue is the continuing disagreement between industrialists and environmentalist groups over whether parts of the human body — including genes and human cell lines — can be patented.

A 'conciliation committee' will meet in Brussels next week in an attempt to bridge the gap that still exists between the Council of Ministers, representing the 12 member states of the EU, which supports such patents, and the European Parliament, which voted in May to oppose them.

The subject of the meeting is a draft directive, drawn up by EC officials but extensively revised in the light of comments from the parliament, which seeks to establish common rules on biotechnology patenting for the member states and to clarify the boundary between what can and cannot be patented (see *Nature* 361, 285; 1993).

The biotechnology industry, which originally supported attempts at harmonization, has already accepted some of the conditions demanded by the parliament. For example, it

maceutical industry, has issued a statement in which it confirms its support for patents on genes and gene fragments "in a form that does not occur in nature". The statement adds: "For example, isolated genes do not occur naturally, nor do large quantities of purified proteins; they should, therefore, be patentable."

This position is directly contested by the European Green Party, which has accused the biotechnology industry of "diminishing humanity in its breathless pursuit of profits", and is asking the European Parliament either to throw out the directive or to insist on its amendment, approved in a vote in May, forbidding the patenting of isolated human body parts.

"What we are talking about here is the commercialization of the human body," Hiltrud Breyer, a member of the European Parliament who represents the German Green party, told a press conference which was held in Brussels earlier this month. "If this directive is adopted [without the amendment], then we will have privatized humanity in the European Union."

While the Green party, as well as supporters from other parliamentary groups, intend to use case histories to back their arguments against patents, representatives of the biotechnology industry claim that the principle of being able to patent genes and human cell lines is so important that they would be prepared to see the whole directive abandoned rather than give way on the issue.

Others point out that, in a series of recent decisions, the European Patent Office (EPO) has demonstrated that it is prepared, in general, to accept the industry's definition of what should and should not be patented under the terms of the European Patent Convention.

If the conciliation committee rejects the parliament's amendment, its decision could, under the terms of the Maastricht Treaty, still be rejected by an absolute majority (that is a majority vote of all members) in the parliament.

Environmentalist representatives such as Sue Mayer of Britain's Greenpeace claim that there would be widespread public support for such action. Accepting the directive without the parliament's amendment, she says, would be seen as giving parliamentary approval to the patenting of genes and other human parts.

But the biotechnology industry is reluctant to submit itself to this particular test of public acceptability. No one will therefore be surprised if this particular effort at harmonizing European patent legislation ends up being quietly shelved. **David Dickson**

Taiwan's 'NIH' begins to bear its first fruit

Taipei, Taiwan. Despite initial suspicions and a lack of legal status, Taiwan's emerging National Health Research Institutes (NHRI), modelled on both the US National Institutes of Health and Britain's Medical Research Council, is already making its mark on medical research.

Officially the NHRI, which aims to have about 500 researchers in its intramural programme within five years, does not yet exist. A new law setting up the institutes has only just passed the first of three stages in the Legislative Yuan. But with strong support from the medical community, it is widely expected to reach the statute books by the end of this year or early 1995.

Furthermore, construction has only just begun of a building that will provide the NHRI with temporary headquarters. These will take up several floors of the new Institute of Biomedical Sciences of Academia Sinica in Nakang, Taipei, before moving to a new site in Taipei in about five years.

But scientists are already lining up to join the intramural programme expected to start next year. And NHRI is having an impact on medical research in Taiwan through its extramural grant programme which began three years ago (see *Nature* 366, 500; 1993). This provides grants of up to NT\$15 million (US\$0.6 million) a year to 39 centres of excellence in universities and hospitals, as well as 25 grants of up to NT\$5 million for individual researchers throughout Taiwan.

Cheng-Wen Wu, the director of the Institute of Biomedical Sciences who is leading the efforts to create the NHRI, says that most of the grant-holders got "really good" reviews in its first external review a few months ago. A further 20 new grants will be added to the programme this year and many existing grants will be extended.

Wu admits that some people were initially "suspicious" of his plans to establish the NHRI. His own institute has grown rapidly to become the largest of Academia Sinica's 21 institutes. Some directors of other institutes are not only jealous of its size, but also sceptical about its output, particularly as the Taiwanese government is restricting research spending because of large outlays for defence.

But Wu claims that he now has the full backing of Taiwan's medical community. The deans of all university medical schools, the heads of research hospitals and Taiwan's medical association and pharmaceutical association are all supporting the legislation to establish NHRI.

Wu expects the intramural programme to get under way in the new building next spring. **David Swinbanks**



has agreed to a condition, inserted after pressure from farming groups, that farmers should be exempt from standard licensing requirements on genetically engineered seeds when producing their own seeds to grow from one season to the next.

But the industry, and in particular various large pharmaceutical companies, remains firm in its conviction that it must be free to patent genes and human cells where these have been isolated and characterized outside the human body. This is necessary, it says, to allow it to profit from new diagnostic and therapeutic treatments.

Interpharma, for example, the association that represents the powerful Swiss phar-

Gravity experiment faces a three year delay

Washington. A revamped proposal for the Laser Interferometer Gravitational-wave Observatory (LIGO) is being backed by the US National Science Foundation (NSF). It will cost 40 per cent more and take three years longer to complete than one endorsed by the science funding agency in 1990.

The increase in planned costs and timescales are being blamed on 'management failures' resulting from the previous running of the project, compounded by Congress holding up funds as a result. Robbie Vogt, a physicist at the California Institute of Technology (Caltech) and LIGO's founding father, was replaced as project manager in March by Barry Barish, a high-energy physicist with experience in managing large parts of the now-defunct Superconducting Super Collider.

Barish is putting together a LIGO management team, including financial controllers and systems engineers, to ensure that the project is built to its new cost and schedule. Previously, he says, "it was organized in the way you would run a laboratory", with a couple of people in charge and no real structure for delegating responsibility.

The National Science Board, the NSF's governing body, agreed last week to support LIGO on the basis of new estimates of \$297 million to build over seven years — the original estimate was \$212 million over four years — and another \$69 million to operate for four years, starting in 1997.

NSF officials will meet congressional staff this week, and are optimistic about securing continued congressional support for the project.

The proposal includes two ground stations, one at Hanford in Washington state, and the other at Livingston Parish in Louisiana. These will use advanced optical techniques to detect the tiny vibrations caused by distant cosmic events.

Neal Lane, director of the NSF, says the decision to proceed "was a very difficult one, because we know that future budgets are tight and that LIGO costs will be higher than originally authorized". Lane refuses to specify which NSF programmes would be cut to meet the extra costs. "It is hard to predict what the trade-off is going to be, but we know there will be a trade-off," he says.

The new money for LIGO includes an extra \$37 million required to cover the extension of the project by the further three years, \$16 million of extra management costs, \$23 million to cover new research and construction costs, and \$9 million extra

for contingencies.

LIGO is being built jointly by Caltech and the Massachusetts Institute of Technology. The extension of the project construction timescale has been caused by actual project management problems, compounded by Congress holding up money until the problems were seen to be solved.

LIGO is an all-US project. But physicists hope that its two detectors will be complemented by two others, one in Europe and the other in Australia or Japan. An earlier plan to build a detector near Hannover in Germany hit problems, but an Italian-French collaboration for a detector near Pisa is well advanced.

Earlier this month, Australian physicists expressed disappointment with the amount of support they will get from their government to plan for a detector there (see *Nature* 372, 121; 1994).

Each LIGO detector will consist of an

evacuated, L-shaped pipe, with limbs four kilometres long and one metre in diameter. The detector will work by reflecting highly coherent lasers off mirrors mounted on free-hanging weights at the end of each limb. When distant cosmic explosions shake the weights by as little as 10^{-16} — one-hundred-millionth of the diameter of a hydrogen atom — interference between the lasers will reveal the movement, scientists say.

Physicists are excited at the prospect of a detector that will either confirm and help to refine Einstein's theory of relativity, or — if it fails to pick anything up — suggest that he was wrong. Astronomers, some of whom feel they already have enough data from optical and radiotelescopes, have been markedly less enthusiastic. But Barish points out that LIGO will open up an entirely new field of astronomy, dependent not on the photon but on its hitherto elusive cousin, the graviton.

Colin MacIlwain

Germany links education to science

Munich. One of the few surprises in Chancellor Helmut Kohl's new cabinet, announced last week, has been the appointment of Jürgen Rüttgers as Germany's new "minister for the future".

Rüttgers takes over the newly created ministry of education, science, research and technology, which will be known as the BMWFT (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie). The ministry is a result of a merger between the former education and research ministries, created out of a desire for smaller government.

Even by German standards, the BMWFT is a bit of a mouthful, explaining why its working title — presumably now to be dropped — was "ministry for the future". The title also emphasized the raised status of the new ministry; former governments have considered education and research as relatively minor ministries.

Rüttgers, a Christian Democrat who is only 43 years old, has been a member of the German parliament since 1987. His star has risen rapidly. Although a lawyer with no previous scientific expertise, he immediately joined the parliamentary research committee (1987–90), and from 1987 to 1989 headed the parliamentary

inquiry committee whose work led to the establishment of an office for technological assessment.

During his first years in parliament he developed a particular interest in space activities, of which he was generally very supportive. In 1987 he wrote a short guide to Europe's options in space technology. He has been parliamentary whip for the Christian Democrats since 1989.

Acknowledged as both highly intelligent and politically astute, Rüttgers' appointment has been welcomed in most quarters. But he is also known to be very ambitious, and newspaper articles have speculated that he may view his new ministry, despite its higher profile, merely as a career steppingstone. If so, he would be following the footsteps of Matthias Wissmann, a former research minister who held office for three months early last year before transferring his portfolio to the ministry for transport.

The BMWFT will have two parliamentary state secretaries: Bernd Neumann, who held this position in the previous research ministry, will be joined by Cornelia Yzer, who has been transferred from the previous ministry for women and youth.

Both Paul Krüger, the former research minister who comes from east Germany, and Karl-Hans Laermann, the former education minister, have left the cabinet. The former environment minister, Klaus Töpfer, widely expected until the last minute to head the BMWFT, becomes minister for urban development. He is replaced by Angela Merkel from east Germany.

Alison Abbott



Rüttgers: minister with a future?

CORRECTION

A recent news item (*Nature* 371, 549; 1994) should have said that the Japanese Meteorological Agency (JMA) is able to provide rapid measurements of the 'intensity' of earthquakes felt in various cities, rather than their 'magnitude'.

Polymerase patent problems

SIR — We are concerned about the broad scope of the European patent provisionally awarded to Hoffmann-La Roche for *Taq* DNA polymerase (see *Nature* 372, 212; 1994). Unlike its US counterpart, this patent does not claim DNA polymerase from *Thermus aquaticus* alone. Instead, it claims all thermostable nucleic acid polymerases, including DNA and RNA polymerases, with relative molecular masses of 86,000–90,000 that show high fidelity.

According to the European patent examiner involved in this case, Roche was able to demonstrate that, in contrast to the previously described *Taq* DNA polymerase¹, its enzyme showed almost no activity in an amplification assay when one nucleotide was removed. This indicates a high fidelity or accuracy in DNA replication, even though the enzyme lacks a 3'–5' exonuclease activity and cannot therefore remove misincorporated bases. Indeed, because *Taq* lacks 3'–5' exonuclease activity, it has been thought unsuitable for applications requiring high fidelity. This has led to the introduction of alternative enzymes such as *U1Tma*, which is sold by Roche's PCR (polymerase chain reaction) partner Perkin-Elmer. It is the demonstration of high fidelity in *Taq* that appears to fulfil the novelty criterion.

The claims made in Roche's European patent cover every polymerase with these characteristics. These include three market competitors of *Taq*, Vent and Deep Vent (*Thermococcus litoralis*, New England Biolabs) and *Pfu* (*Pyrococcus furiosus*, Stratagene). Lundberg² demonstrated that *Pfu* has an 11–12 fold greater replication fidelity than *Taq*, with an average error rate of 1.6×10^{-6} for *Pfu* and 2.0×10^{-5} for *Taq*. Kong *et al.*³ showed that Vent has a K_m of 0.07 nM compared to 1.4 nM for *Taq*. A low K_m value indicates an enhanced ability to replicate low DNA concentrations efficiently. Literature from New England Biolabs⁴ indicates an error rate of 57×10^{-6} for Vent compared to 285×10^{-6} for *Taq*. In the case of Vent, Deep Vent and *Pfu* it has always been thought that the 3'–5' exonuclease activity is responsible for the low error rates, yet Roche has apparently managed to demonstrate sufficiently high fidelity of *Taq* for these enzymes to be covered in the patent claims. If the broad scope of Roche's European claims is upheld, these existing products will be in conflict with the new patent.

Our unease does not end here, as this patent could be used to prevent the sale of polymerases yet to be discovered. Because of this potential, the European patent examiner is expecting a large number of objections. Paradoxically, the latest

innovation in PCR (long-range PCR)⁵ uses a combination of DNA polymerases, a technique that makes use of alternative enzymes developed by other companies in the absence of broad patent protection for nucleic acid polymerases. We have here another clear example⁶ of an undesirably broad patent that provides the applicant with a monopoly which may inhibit the development of new and better products.

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Opting for silence

SIR — John Funder finds it neither correct nor attractive to silence the 'p' when pronouncing words in which the Greek-derived 'pt' occurs in the middle of a composite word (*Nature* 371, 98; 1994). 'Helicopter' is, he believes, a convincing example. Undeniably, helicop-ter is the universally accepted pronunciation, but at the same time one has to realize that few people reflect upon the etymology of this word. However, in names of compounds such as aminopterin, an amino derivative of pterin, one must clearly opt for silencing the 'p' (as in apoptosis) in order to secure the understanding of the underlying chemistry.

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SIR — In writing about the etymology of the word apoptosis, Funder misses an important distinction between pronunciation and spelling. Indeed, using Funder's own examples, he shows when the 'p' is pronounced and when it is silent.

Apoptosis, as in pterydactyl, has a 'pt' at the beginning of a syllable. Unlike helicopter (or optic, option and so on), the 'p' is silent. Such a distinction is also made when a 'p' is followed by an 's', as in psychology. Even when the 'p' is preceded by an 'o', as in neuropsychology, the 'p' is silent.

An argument could be made that the

word is pronounced a 'pop'tosis, where the -pt would be in the middle of a syllable. However, that would be denying the etymology of the word on which Funder based his own argument. Because the term used to describe a particular type of cell death is derived from two greek words, *apo-* and *-ptosis*, the syllabic break is made between the two words when they are joined. Thus apo'(p)tosis is the correct pronunciation.

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Nurture not nature

SIR — Although the alleged taking of a pink pigeon, now presumed dead, by a Mauritius kestrel is unfortunate, you go too far in describing the outlook for species preservation as "gloomy" (*Nature* 371, 544; 1994).

True, in the short term, greater protective measures will have to be taken; however, in the long term, the solution certainly lies in improved education. Perhaps, in the effort to re-establish native populations, insufficient attention has been paid to inculcating proper respect for species with divergent life-styles.

It is well to remember that the kestros-Mauritians (as they are properly termed) have, over the past century, suffered a devastating and nearly complete loss of their cultural as well as their genetic heritage at the hands of the very species who would now save them. Is it, then, surprising that these proud birds, who, after all, give the Ile aux Aigrettes its name, should strike out, in revenge, at the weak?

While it would be naive to suppose that this problem will disappear either quickly or completely, I am confident that it will, in time, yield substantially to the same sorts of efforts to which so many of our once-pressing social ills have proved susceptible: sympathetic counselling by both trained professionals and peer ex-offenders, the establishment of support groups for those attempting to overcome their habituation to this form of violence, and, most importantly, exposure, from an early age, both in the nest and in the classroom, to the simple truth that, when all is said and done, "under the feathers, we are all birds."

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The WHO and why of HIV vaccine trials

John Moore and Roy Anderson

A World Health Organization (WHO) advisory committee meeting in Geneva at the end of last month approved phase III trials for HIV vaccines in developing countries. What is the justification for this decision?

WHY has a WHO committee, of which we were members, just approved HIV vaccine testing in developing countries (see *Nature* 371, 644; 1994) when the National Institutes of Health had earlier decided not to go ahead with trials in the United States? The approval was given for phase III trials (efficacy studies in communities where HIV is being transmitted) of recombinant subunit HIV envelope-protein vaccines which are not being undertaken in the United States because of uncertain efficacies of the available gp120 candidate immunogens. In our view, the two decisions are not inconsistent, because the circumstances influencing the desirability of a phase III trial in the United States (where HIV infection is still largely restricted to high-risk groups) and parts of the developing world (where the prevalence of infection in pregnant women attending antenatal clinics in urban centres has reached 20% or more) are quite different.

Of the 16 million people that are estimated by WHO to have acquired HIV infection, 90% live in poor communities in the developing world. Despite these chilling statistics, which highlight the urgent need for an HIV vaccine, some may interpret the WHO decision as the result of pressure by biotechnology and pharmaceutical companies who wish to experiment with products deemed unsatisfactory for use in Western countries. But this would not be a fair reaction to the WHO decision. The WHO committee was not short of information (five biotechnology companies made presentations, two of which, Biocine and Genentech, have products available for phase III trials) and contained a broad range of expert opinion, including participants from developing countries and representatives of 'at-risk' communities. There remain many scientific uncertainties, but the committee's decision was informed and objective.

Decisions

What are the uncertainties and what were the key issues that influenced the decision? The urgent need for safe and effective HIV vaccines and the desirability of conducting vaccine-efficacy trials in communities with high rates of transmission were not in question. Vaccines are a major success story of biomedical research in the twentieth century, given the global eradication of smallpox and the successful control, at least in some parts of the world,

of nine major infections — tuberculosis, diphtheria, tetanus, yellow fever, pertussis, polio, measles, mumps and rubella. New recombinant vaccines for *Haemophilus influenza* (serotype b — Hib) and hepatitis B virus are beginning to have a very significant impact on incidence in areas where they have been used in mass campaigns. In most of these cases, however, one factor is of major importance — the comparative genetic, and in particular antigenic, stability of the infectious agent in different parts of the world. Attempts to develop vaccines with high efficacy (for example protecting 80% or more of those vaccinated), have so far failed for pathogens that exhibit marked antigenic variation both within and between hosts.

In many respects the malarial parasite, *Plasmodium falciparum*, is a good paradigm for HIV. It exhibits great genetic (antigenic) variability, is highly pathogenic and in global terms is a chief cause of human mortality. Further, the effector mechanisms inducing acquired immunity are poorly understood, and much controversy surrounded the initiation of phase III clinical vaccine trials in the developing world with an immunogen of uncertain efficacy. The results of randomized double-blind placebo-controlled trials in Tanzania in a region of intense malaria transmission of a synthetic polypeptide candidate vaccine (SPf66) against clinical malaria have now been published (P. L. Alonso *et al.* *Lancet* 344, 1175–1181; 1994). The study confirmed that SPf66 is safe and immunogenic but, more important, it revealed an efficacy of 31% (confidence interval 0–52%) in 274 children aged 1–5 years who received three doses of vaccine. Given this level of efficacy, the potential of the SPf66 vaccine as a public-health measure in Africa will be much debated in the coming months. However, as falciparum malaria causes between 1 and 3 million deaths per year, largely in young children and pregnant women in sub-saharan Africa, a vaccine with a moderate-to-low efficacy is, without doubt, a valuable addition to existing control measures.

The debate about the HIV vaccine trials among the WHO committee members centred on several issues, including the likelihood that the immunogens might enhance disease progression and perhaps infectiousness in recipients who subsequently became infected, and that they could increase the rate of HIV transmis-

sion by inducing a feeling of safety in recipients, resulting in increased high-risk sexual behaviour. The latter argument is important, and careful counselling will be essential in any clinical trial, but concern over behaviour change has not prohibited the use of chemotherapeutic agents that do not eliminate HIV but prolong infectious lifespan in those treated.

The greatest concern of members of the WHO committee, however, was that the products simply would not work, in the sense that the degree of protection they conferred to an individual would be so slight as to be insignificant on a population basis. This view tended to be held by researchers who had studied the immune response to subunit vaccines in humans and animal models, whereas clinical scientists and epidemiologists were generally less swayed by uncertainties over which immunological markers best correlated with protection, or disagreements about the relevance of results from HIV-1 infection in chimpanzees to infection and disease in humans. Clinicians and epidemiologists argued that the only way to determine efficacy is to run an efficacy trial, which is both true and a truism, and that failure is an acceptable outcome. Others disagreed, and felt that the chances and consequences of failure are sufficiently great that a phase III trial of the recombinant gp120 candidates is unwarranted.

Herein lies the crux of the debate: the likelihood of the products being efficacious to any extent. Unfortunately, nobody knows with any certainty, and it all boils down to a matter of opinion. Nobody, not even the most passionate advocate of a trial, believes that efficacy would approach 100%, and few at the meeting were prepared to guess at even 50%. A significant minority felt that efficacy would be close to 0%. They therefore argued that the available recombinant gp120 products were not suitable for a phase III trial — but they could not be certain that their 0% estimate was right. Thus, opinion was strongly divided about the efficacy of the vaccine candidates, leading to many modifications to the final statement released by WHO before all members of the committee could endorse it.

A key point in the discussion was whether a low-efficacy vaccine would be of benefit to countries where HIV is spreading rapidly both in high-risk groups and in the general sexually active popula-

tion. It is difficult to answer this question with precision, given the many uncertainties that surround the properties of the immunogens and the factors that control transmission in a given community. Mathematical models of the transmission dynamics of HIV provide a crude template with which to explore this issue. Analyses presented at the meeting suggested that even efficacies of 30 to 50%, with a 5-year protection following vaccination, could save many lives in communities in which transmission rates are high. Here lies the analogy with the recent malaria vaccine trial referred to above, in which efficacy was found to be 31%. But achieving this degree of protection with an HIV immunogen may be very difficult, given the enormous genetic diversity of the virus and its continued, rapid evolution as it spreads locally and worldwide.

A further issue of relevance concerns the cost of a product to be used on a community-wide basis. Cost is a central question for all mass-immunization programmes, particularly for those in developing countries, which have limited resources for health care. At present, little is known about the likely cost of an HIV vaccine, but this issue will become of paramount importance if efficacy is low and duration of protection short. Preliminary studies based on mathematical models of HIV transmission suggest that the most beneficial vaccination programme design, in terms of minimizing the cumulative number of cases of infection in areas of intense transmission, is blanket coverage of the sexually active population, repeated annually in an unselective manner (irrespective of a previous history of vaccination). This, combined with the need to monitor continually the most appropriate immunogen(s) on which to base the vaccine due to the continuing evolution of the virus, suggests that costs of any long-term immunization programme will be high. Similar issues pertain for the new malaria vaccine.

Recommendations

So what exactly was recommended by the WHO committee? Crucially, it concluded that any decision to go ahead with a trial of any product must be made by the government of the country hosting a trial, and that phase I/II safety trials in that country should precede a phase III trial. This will, of course, expose various governments to extensive lobbying over the next few months from vaccine manufacturers and clinical-trial coordinators. But the governments concerned will have the full support of the WHO when they make a decision that is most suitable for their populations, taking into account the benefits and chances of success, and the consequences of failure.

The WHO committee stated that the particular HIV-1 subtypes circulating in

the proposed trial population should be a major consideration; it was felt, for example, that a vaccine based on a subtype B envelope should not be tested in an African country where viruses of subtypes A and D predominate. Counselling of the trial populations as to the potential risks of vaccination and the necessity of following safe sexual practices was agreed to be mandatory; indeed, an increase in the amount of information provided to a trial population was considered by some committee members likely to have a greater impact on HIV transmission than the vaccines themselves.

This particular concern highlights a further problem — the design of a phase III trial. The numbers of people required in each arm of the trial (treated and placebo) is related both to expected efficacy of the vaccine and to the rate of HIV infection in the trial community. The latter parameter changes over time in the absence of any intervention, moving from low to high to low again as the epidemic develops. The former is difficult to predict. Both factors mitigate toward trials involving many thousands of treated individuals (perhaps tens of thousands).

Most eyes are now unofficially focused on Thailand, where the characteristics of the current HIV-1 epidemics are widely thought to be favourable for a phase III efficacy trial. Two genetically and antigenically quite divergent subtypes of HIV-1 are present in different Thai sub-populations: subtype E is transmitted predominantly by heterosexual contacts in northern Thailand; whereas subtypes B and E both circulate among injecting drug users in Bangkok. However, many of the subtype B viruses found in Thailand are somewhat divergent from the MN and SF-2 North American strains used to make the current gp120 subunit vaccines.

The above cohorts are the potential targets for separate efficacy trials, each of which has advantages and disadvantages. The favoured test population for the presently available subtype B vaccines is the Bangkok drug users, to address the issues of inter- and intra-subtype efficacy simultaneously. However, preliminary results presented earlier this month by Marc Girard and Patricia Fultz at an AIDS vaccine conference in Washington DC, showed that a chimpanzee infected with a subtype B virus was easily superinfected on challenge with a subtype E virus. Although the WHO committee had significant reservations about the chimpanzee model, it is hard to imagine that a vaccine-induced response to a subtype B gp120 would be superior to the infection-induced response to a subtype B virus in protecting a chimpanzee or human from infection with a subtype E virus.

A second concern about a trial in the drug-user cohort is that the success or failure of a gp120 vaccine against virus

transmission via injected blood may not necessarily predict success or failure of a similar vaccine in a population where transmission is predominantly sexual — in other words, in the vast majority of the cases of HIV-1 transmission worldwide. Thus, a trial in a drug-user cohort might need to be repeated in a sexual-transmission cohort, whatever its outcome.

A potentially smaller and simpler trial would involve a subtype E gp120 vaccine in the northern Thailand sexual transmission cohort. This trial might not address inter-subtype protection, but this would only be relevant if there were any significant intra-subtype efficacy, which is a far from certain outcome of any trial. Although no subtype E gp120 is yet available for an efficacy trial, there are apparently plans to make such a protein ready in a year or so. Although the WHO committee made no specific recommendations, we believe that if an efficacy trial is done at all, the best option is the simplest one: a subtype E gp120 trial in the cohort where strains of predominantly subtype E are transmitted sexually.

One of the most important statements made by the WHO committee was to emphasize a long-term commitment to AIDS vaccine development and testing worldwide. All of us, government and community representatives, academic and corporate scientists, welcome this support for something to which we are all deeply committed: the eventual development of a globally effective AIDS vaccine or vaccines. The present generation of gp120 subunit vaccines was designed around 1984; in 1994 there is a clear need for better products given the expectation of low efficacy in phase III trials with the currently available vaccines. But the desire for a vaccine is universal, and the need for more national and international support for further fundamental research on the relevant topics has been clearly demonstrated.

The eyes of the world are on HIV vaccine researchers, today more than ever. The results of the development and the recent trials of the malarial vaccine hold many important lessons for the HIV research community. A consensus has been reached by the WHO in good faith: to leave a decision for a phase III trial of HIV subunit vaccines to national governments, and to support those who choose to do so. These governments are quite capable of deciding in their own best interests, so perhaps it is time for us all to leave them to do so. □

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Problems of more than one electron

The spectrum of helium and other atoms with two electrons has been a bother since the early days of quantum mechanics. And there are perplexing issues outstanding still.

THE darkest days of the quantum theory must have been the decade or so between the appearance of Bohr's semi-classical theory of electron orbits and the first appearance of modern quantum theory more than a decade later. Bohr had given a persuasive account of the hydrogen atom, accounting for the regularities of the Periodic Table in the process, but despite the efforts of people such as Sommerfeld, nobody could make much sense of the measured spectrum of helium, the simplest electron configuration after hydrogen. Was Bohr's account of the hydrogen atom flawed in its assumptions?

By the mid-1920s, after Heisenberg and Schrödinger, the origin of the difficulty (but not its resolution) must have been plain. With helium just as with hydrogen, there will indeed be a series of states in which a helium atom can exist, but calculating them from first principles is a bother because of the need to take account of the electrostatic repulsion between the two electrons.

Even enumerating the states accurately is not straightforward. The obvious classifiers to use are the total angular momentum of a state and its projection along some axis, but who could guess which one-electron states must be mixed together to make the states the spectroscopists observe?

Formally, there is no great difficulty. Simply take a complete set of wavefunctions representing electrons in a hydrogen atom, mix them all together with undetermined coefficients, substitute that mixture in the relevant Schrödinger's equation and solve the resulting linear algebraic equations. There are two important snags. Experience has shown that it is necessary to use hundreds, even thousands, of wavefunctions to get reasonably accurate results — and the size of the matrix equation that must be solved is identical with the number of wavefunctions, say M , used as a starting point.

The second and more serious difficulty is that somebody has to evaluate $M(M-1)/2$ integrals, one for each distinct pair of the basis functions, in which the integrand is the product of the pair of wavefunctions, each of which is a function of the space coordinates of one or other of the two electrons in a helium atom; that product is then divided by the geometrical distance between the two coordinate points and the integration is carried out over all six coordinates from zero to infinity. Even when it is commonplace to use simplified wavefunctions in the calculations, who can wonder that serious attempts at the accurate calculation of atoms beyond hydrogen began only in the 1970s (when

computers began to make their mark)?

Now, there seem to be two approaches to the problem: computational and reflective. S.P. Goldman from the University of Western Ontario, seems to have struck oil in the search for better ways of computing electronic states (*Phys. Rev. Lett.* **73**, 2547–2550; 1994). For one thing, he has found a neat way of dealing with the especially brutish feature of the integrals occurring in these problems, which consists of a cusplike feature in the electrostatic potential when the two electrons are equidistant from the helium nucleus and which comes about because the potential energy would be literally infinite if the two electrons occupied the same point. He has also modified the standard set of simplified wavefunctions due to J.C. Slater to give the inner and outer electrons different distributions in space.

The immediate result is that many fewer wavefunctions are needed to win accurate results. Goldman reckons to have calculated the energy of the ground state of helium to within 4.6 parts in 10^9 , at least three orders of magnitude better than previous results, by using "only" 305 starting functions. For good measure, he used only a desk-top workstation for the arithmetic.

The reflective one is Michael E. Kellman from the University of Oregon at Eugene, and almost literally so. Not often, these days, is an author given space to rehearse his past contributions to the literature of his subject, but Kellman seems to have won that privilege from a journal notoriously tightfisted with paper (*Phys. Rev. Lett.* **73**, 2543–2547; 1994). But the circumstances are exceptional; Kellman has had an important afterthought 20 years after the event.

He and D.R. Herrick were pioneers in the 1970s of the pursuit of the two-electron atom and its spectra. Unlike many others, they had a physical model to guide them. As electrons repel each other, will they not tend to be found on the opposite sides of the nucleus? That makes a helium atom a kind of triatomic molecule, but one in which the supposed bonds holding the outlying electrons to the nucleus are simply their mutual electrostatic repulsion. So the bonds, on this analogy, have very small force constants.

Herrick, Kellman and their contemporaries in the early 1970s made much of this analogy. Vastly improving on what Sommerfeld has attempted half a century earlier, they were able to conjecture what states would arise in helium if each of the electrons were excited above the ground state, not necessarily to the same degree.

They predicted multiplets of states and groupings thereof (called supermultiplets), finding confirmation in the data.

And the afterthought? It should be possible to classify the states of a two-electron atom by the choice of an appropriate symmetry group. That is how group theory, without the intervention of conventional algebra, can by itself be used to show that the states of one-electron atoms are precisely those found in the laboratory or by solving the appropriate Schrödinger equation algebraically.

In the 1970s, Herrick and Kellman were already guessing that the three-dimensional rotation group that accounts for the properties of the angular momentum of one-electron orbits would not suffice for their floppy triatomic molecules, and were guessing that they would have to use the four-dimensional orthogonal group called $O(4)$ instead. The use of the three-dimensional analogue, $O(3)$, which works well enough for one-electron atoms, leads directly to the slow convergence and poor approximation that Goldman is seeking to circumvent.

The more tangible part of what Kellman has now done is to apply the formalism of classification by $O(4)$ to cases in which, in the approximation in which the classification at first ignores the electrostatic repulsion between electrons, two electrons have different principal quantum numbers. More multiplets and supermultiplets fall out of that. It turns out that the classification is best represented by quantities such as the momentum (quantized, of course) locked up in the bending vibrations of the floppy triatomic molecules, which quantities function as quantum numbers for the multiplets and supermultiplets. People working in the field will be waiting eagerly for the details.

Meanwhile, there is a puzzle: the $O(4)$ group, or more strictly its unitary equivalent $U(4)$, keeps throwing up extra terms, which seem not to fit in well with the classification of the states of two-electron atoms and which have no obvious physical meaning. Could they represent some collective motion of two electrons bound to a nucleus that nobody has yet thought of? Kellman thinks that unlikely, but there is no other obvious way round the difficulty. Is this one of the rare cases in which mathematics has failed to be ready with a solution to physicists' problems? Or is the model of the triatomic molecule inappropriate? The conjunction of the two papers by Kellman and Goldman will send many people scurrying in search of an explanation.

John Maddox

Mice sans synaptotagmin

Erwin Neher and Reinhold Penner

In the past two years we have come a long way in the biochemical characterization of synaptic proteins. It has emerged that some 6–8 of them interact to form the so-called fusion complex (see refs 1–3 for review); and that homologues of some of the proteins are located at different places in the cell interior, wherever intracellular membranes are believed to fuse with one

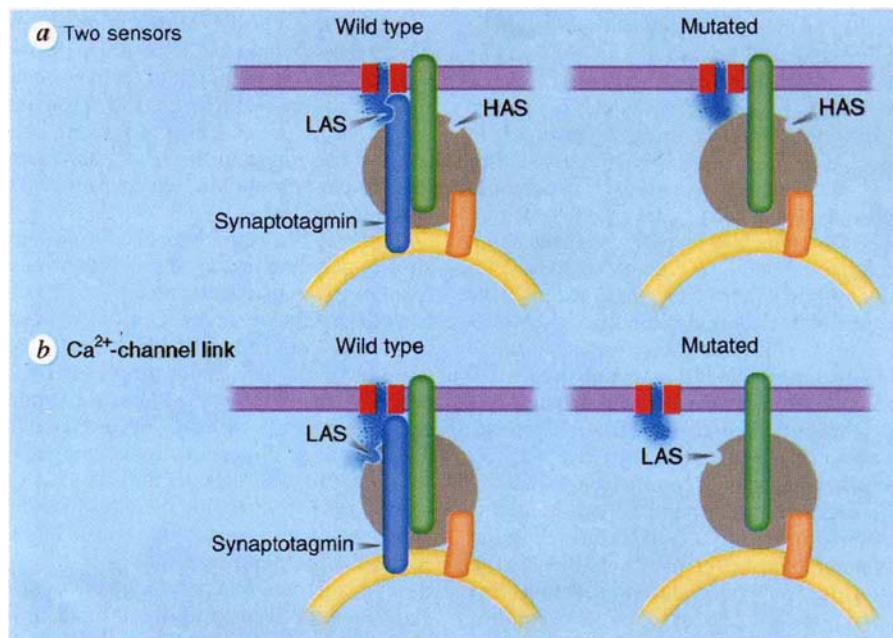
arrested at the 'active zone' (the site of neurotransmitter release) until an action potential arrives and increases the concentration of Ca^{2+} . It was therefore argued that synaptic vesicles possess a 'fusion clamp' which prevents fusion otherwise. Synaptotagmin, by the mere fact of its presence at sites of regulated fusion, was held up as a candidate for carrying out the

tagmin gene are phenotypically indistinguishable from heterozygotes or wild-type mice. Transgenic animals breathe normally, and respond to tactile stimulation; the retina and brain structure show no morphological abnormalities. If one believes that synaptotagmin I is central to neurotransmitter release, and that some functional interaction is required for establishment of a correct body plan, these findings are quite unexpected.

Nevertheless, Geppert *et al.* find that synaptic transmission in cultured hippocampal neurons is severely perturbed, but in a more subtle way than would be expected if there were just a single Ca^{2+} -sensor. They recorded from pairs of hippocampal neurons forming contacts in cell culture, stimulating the 'presynaptic' cells and then analysing responses in voltage-clamped 'postsynaptic' cells. With wild-type cells, large-amplitude, postsynaptic currents were observed, which were well synchronized with presynaptic action potentials. A slower 'asynchronous component', lasting some 100 milliseconds, contributed only little to the overall signal. In cells from mutant mice, on the other hand, the fast synchronous component was almost completely absent. The asynchronous phase, however, was unaltered or slightly increased.

Geppert *et al.* interpret the data in the light of a study by Goda and Stevens⁹, which describes two such components of neurotransmitter release: a fast component mediated by a highly efficient, low-affinity Ca^{2+} receptor; and a slower component mediated by a less efficient, high-affinity receptor. In this scheme, synaptotagmin would be the low-affinity sensor, which efficiently elicits secretion during the short period of the action potential when concentrations of Ca^{2+} at the release site reach very high levels (see left-hand part of *a* in the figure). Mutant mice lacking synaptotagmin (right-hand part of *a*) would be stimulated only through the inefficient (but high-affinity) receptor; they therefore show only a small, slow response, while residual Ca^{2+} , after having spread out diffusively, decays back to basal levels.

This proposal explains the data very well, and provides a detailed picture of the events preceding fusion. But other interpretations are possible. One is offered by the so-called Ca^{2+} -voltage hypothesis¹⁰, which holds that transmitter release is regulated both by a Ca^{2+} -sensor and by a voltage sensor. It seems that the simple postulate that synaptotagmin is the voltage sensor would, by and large, explain the results (we are aware that its molecular properties appear to be incompatible with this assumption, but it might be coupled to a voltage sensor). A study on synaptotagmin-deficient PC12 cells can be interpreted in terms of the Ca^{2+} -voltage hypothesis, however, with the postulate



Models for the role of synaptotagmin in eliciting vesicle-membrane fusion. Each of the four diagrams shows a vesicle attached to the plasma membrane by a fusion complex. *a*, The two-sensor model⁹. Two Ca^{2+} -sensors, a low-affinity one (LAS) on synaptotagmin and a high-affinity one (HAS), respectively provide for fast synchronous and asynchronous release. The LAS normally senses short intensive pulses of Ca^{2+} when Ca^{2+} -channels open, but not in the absence of synaptotagmin (right). The HAS is not necessarily a single binding site, but may represent several Ca^{2+} -dependent steps preceding fusion. *b*, A speculative, alternative model, with only one low-affinity Ca^{2+} -sensor. Here, synaptotagmin's function is to link a Ca^{2+} -channel to the fusion machinery. In its absence (right), channels and release sites are randomly distributed, so that the Ca^{2+} -sensor no longer experiences short intensive pulses of Ca^{2+} .

another. Synaptic proteins are conserved between man and yeast, so there is no doubt that the fusion complex is one of the very basic molecular machines. It not only mediates synaptic transmission (by letting synaptic vesicles fuse with the plasma membrane) but also enables membranes to flow from their site of synthesis to the cell surface through various cycles of vesicle formation and fusion.

It seems, then, that we know which players take part in this game. But what does each of them do? A paper by Geppert *et al.*⁴, published in *Cell* last week, provides some clues to help start assigning specific functions to specific proteins.

Fusion of transmitter-containing vesicles during synaptic transmission is precisely regulated by calcium, in contrast to 'constitutive exocytosis' which goes on continuously. Synaptic vesicles seem to be

clamping function. When it was found that each molecule has two so-called C2-domains (which in protein kinase C bind calcium), the obvious conclusion was that synaptotagmin is the Ca^{2+} -sensor of regulated secretion — or, more precisely, a Ca^{2+} -controlled fusion clamp⁵. Geppert *et al.* have now looked at hippocampal neurons from transgenic mice lacking functional synaptotagmin I, one of the two isoforms of the molecule. They conclude that synaptotagmin I is one of the main Ca^{2+} -sensors for transmitter release.

Mutational studies on *Drosophila*^{6,7} and *Caenorhabditis elegans*⁸ provided the initial indications that synaptotagmin is essential for neurotransmission, and that lack of the molecule leads to severe dysfunction in secretion. But Geppert *et al.* find that, immediately after birth, mice homozygous for the knocked out synapto-

WHITE DWARFS

Plugging the generation gap

Harry L. Shipman

that synaptotagmin is the Ca^{2+} -sensor rather than the voltage sensor (W. Huttner, personal communication).

Another (admittedly speculative), explanation is depicted in *b* in the figure. Here, synaptotagmin links the fusion particle and Ca^{2+} -channels. Although synaptogenesis and vesicle docking appear to be normal in Geppert and co-workers' mutant mice, it still may be that, unlike the situation in wild-type mice, Ca^{2+} -channels are not recruited to the molecular fusion complex. The lack of a well synchronized response might then be a simple consequence of the fact that the Ca^{2+} -sensor (which in these circumstances can be a single-type, low-affinity sensor) is not exposed to the extremely high local concentrations of Ca^{2+} which are thought to occur in microdomains at the mouths of open channels^{11,12}. The small component of asynchronous release could then be the response of the same sensor to more generalized but smaller increases in Ca^{2+} , which come about through the overlap and diffusional spread of microdomains.

From its molecular properties, synaptotagmin looks like a Ca^{2+} -sensor, but it may also be important for structural organization. The synaptotagmins bind to neurexins which are involved in many aspects of synapse organization¹³. Also, synaptotagmin I interacts with the clathrin-assembly protein complex AP2 (ref. 14), meaning that it may take part in endocytosis following exocytosis. Last — but not least — synaptotagmin interacts with N-type Ca^{2+} -channels *in vitro*¹⁵.

It will be interesting indeed to find out which of the properties of this multifaceted molecule is responsible for its involvement in exocytosis, and what exactly its Ca^{2+} -sensing ability is used for. Geppert *et al.* have provided some helpful pointers to that end. □

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RESULTS published this month by Martin Barstow and colleagues¹ provide a clue to a long-standing mystery in stellar evolution: the genesis and life cycles of white dwarf stars. The trouble has been that hydrogen-rich white dwarfs are rather cool (in stellar terms), whereas their supposed progenitors, the hydrogen-rich 'planetary nebulae' stars, are extremely hot. Barstow *et al.* may have found the star that plugs the gap. The object known as RE1738+665, identified by the Wide Field Camera on the Rosat spacecraft, is the hottest hydrogen-rich white dwarf yet detected.

Most stars end their lives as white-dwarf stars, tiny balls no larger than the Earth. So, if you like, you can interpret the study of white dwarfs as a part of the study of stellar old age, or stellar geriatrics. White dwarfs are interesting in their own right, as in many respects they are much more varied than are their ancestors on the main sequence of stellar evolution. Some of the highest stellar temperatures, strongest magnetic fields and most unusual photospheric compositions are found in the bottom of the Hertzsprung–Russell diagram of luminosity against temperature, the place where these little stars are found. The life cycles of white dwarfs is, or should be, simple. Because nuclear reactions (for the most part, hydrogen burning) have ceased in their cores, they cool into oblivion.

When white-dwarf spectroscopy began in earnest in the 1950s, it became clear that, broadly speaking, white dwarfs are divided into two kinds. One group has photospheres that show hydrogen lines and little else in their spectra; these are called DAs because their spectra roughly resemble those of the main-sequence A stars. The second group's photospheres show little more than helium lines, and are classified into different spectral categories depending on whether the helium lines are He II (DO), He I (DB) or non-existent (DC, DQ or DZ)². There are many additional categories because some white-dwarf photospheres are polluted by species such as carbon, calcium, magnesium and iron. The circumstance requires some additional explanation because the surface gravities of white dwarfs are so large that the lightest constituent in their outer envelopes should float to the top, and everything else should sink out of sight.

A number of us have been trying to make sense of this complex picture for years, but even the broad question of why white dwarfs are divided into DAs and non-DAs has eluded us. Most of us thought that this distinction pre-dated the white-dwarf stage of a star's life cycle and

that white dwarfs cooled through two channels: the DAs and the non-DAs. But evidence for this picture was slim. In the late 1980s, Gilles Fontaine, François Wesemael and Jim Liebert, three leaders in the field, stunned the rest of us by proposing a single-channel scheme for white-dwarf chemical evolution. This model posited that there was really only one kind of white dwarf, which changed its face as it cooled (see for example refs 3,4). Hydrogen-rich DAs turned into DBs, which then turned back into DAs, changing their costumes like an old-time vaudeville actor. The thin hydrogen skin disappeared when it was mixed with the helium interior; then these helium-surfaced stars changed their faces when hydrogen floated to the top. This proposal explained a number of puzzling features of white-dwarf temperature distributions. For instance, at that time no DA white dwarfs were known with temperatures exceeding about 65,000 K, whereas there were plenty of non-DAs that had such extreme temperatures.

This proposal stirred up a commotion because it required that the hydrogen layer in all white dwarfs was a thin skin on the top of a mainly helium star, somewhere between 10^{-7} and 10^{-14} solar masses depending on exactly what you wanted the single-channel scheme to explain. But our basic understanding of the life cycles of red giants, the stars that turn into white dwarfs, indicated that the hydrogen-layer masses were much higher, about 10^{-4} solar masses⁵. How could a star manage to get rid of almost all, but not absolutely all, of its hydrogen? At least one investigator found the idea so nonsensical that he stopped working on white-dwarf stars for a while. But the single-channel model apparently explained a lot of things, including the extreme ultraviolet photometry of white dwarfs, the 'DB gap' or complete absence of DB stars with temperatures between 30,000 K and 45,000 K, and the pulsational properties of many white dwarfs. It attracted considerable support.

Enter Barstow and colleagues. The Wide Field Camera on board Rosat discovered 384 objects, and measured their extreme ultraviolet (EUV) count rates in two bands, 60–140 Å and 114–200 Å. Some of these sources were known from other catalogues, but some, including RE1738, were not. Spectra of the blue and red dots which sat within the Rosat error boxes were then obtained with ground-based optical telescopes. The nature of RE1738 prompted Barstow's team to obtain an ultraviolet spectrum from the International Ultraviolet Explorer (IUE).

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Analysis of these data showed that RE1738 was an extremely hot, hydrogen-rich, DA white dwarf, with a temperature of 88,000 K. This temperature is way above the previous limit to the DA cooling sequence, which was about 70,000 K. One of the chief arguments in favour of the single-channel model was that the ultrahot DA stars, which should connect the DA cooling sequence with the hydrogen-rich stars found in the nuclei of planetary nebulae, were simply not there. RE1738 thus fills an important gap in the life cycles of cooling white dwarfs.

Because Barstow's team has spectra of RE1738 from 1,200 to 7,000 Å, and photometry in the EUV, they can determine further details on this intriguing star. The EUV and X-ray flux is lower than it should be if the atmosphere were pure hydrogen. As in other hot DA white-dwarf stars, the missing source of EUV opacity cannot be helium, because there is no helium in the IUE spectrum. Specifically, the number density of helium is less than 2.5×10^{-4} times that of hydrogen. Presumably the opacity comes from heavier elements, which astronomers insist on calling 'metals'. The EUV spectra of other hot DA white dwarfs show that some strange cocktail of 'metals' is undoubtedly the missing opacity source^{6,7}.

Does the discovery of RE1738 mean that the single-channel model for white-dwarf evolution is dead? No, not completely dead. This one object fills the gap between the DAs and their progenitors, so it seems clear that a lot of DAs begin their lives as DAs, cool as DAs, and stay that way for a good long while. But there may be another group of stars which make up the non-DAs, and they may mix and change composition as they cool. There are still a number of very odd things about the temperature distribution and number statistics of the non-DAs. One of the strengths of the single-channel model was that it gave us proposals to test; before it appeared we were floundering in the dark. So although RE1738 is one of the most important white dwarfs to be discovered in years, it provides answers to only some of the puzzling questions of white-dwarf chemical evolution. □

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A crash course in survival

Linda Partridge and Mike Bruford

A MARKED decrease in fitness as a result of inbreeding in captive animal species is a commonplace observation^{1,2}. Direct evidence for inbreeding depression in nature has proved more elusive, no doubt at least in part because its occurrence has led to the evolution of mechanisms preventing matings between close relatives³. Studies of a song sparrow population by Keller and co-workers reported on page 356 of this issue⁴, and of a reintroduced popu-



Lukas Keller

Subject of study — *Melospiza melodia*, the song sparrow resident on Mandarte Island, British Columbia.

lation of white-footed mice described last month by Jiménez *et al.* in *Science*⁵, provide two of the most convincing examples to date of the deleterious effects of inbreeding on survivorship in natural environments.

Long-term studies of island populations have proved particularly profitable for investigating ecological and genetic contributions to survival and reproductive success which determine fitness⁶. In the study of Keller *et al.*, the population of song sparrows, resident on Mandarte Island, British Columbia, fluctuated from 10 to 140 breeding individuals, a feasible number of birds on which to obtain individual survival and breeding records. The wide variation in numbers was due mainly to two population crashes associated with severe conditions in the winters of 1979–80 and 1988–89. By the time of the second crash, the mating patterns of all the birds had been recorded in the previous generations. The pedigree for each individual could therefore be used to calculate how inbred it was. During the crash, the more distantly related the parents of a bird, the higher that bird's probability of survival, resulting in an immediately post-crash

population of less inbred individuals.

Jiménez *et al.*⁵ investigated the effects of controlled inbreeding, in the laboratory, on the survival of white-footed mice when they were released into nature. Based on recapture data for marked individuals, the weekly survivorship of the inbred mice was only 56 per cent of that of the non-inbred control individuals. Furthermore, only the inbred mice lost body weight throughout the ten-week sampling period. Most intriguingly, the design of the study allowed comparison of the effects of the same level of inbreeding in the laboratory and in nature. The increase in death rate from inbreeding was much higher when the mice were released than when they were maintained in the laboratory, meaning that data from captive animals may lead to an underestimate of the extent of inbreeding depression under the more stringent conditions of nature.

Given the intermittent selection against inbred individuals in the sparrow population, it might be expected that the deleterious, mainly recessive mutants that are probably largely responsible for inbreeding depression would have been eliminated. But immigrants from the mainland, although on average only one a year in number, may have reintroduced at least some of the variants. In addition, mutant copies could have survived sheltered from selection in heterozygotes. The very small population size after the crash resulted in a marked increase in inbreeding in the ensuing generation, and it would be interesting to know if there were effects of inbreeding depression on survival or fertility then. Increased matings between relatives as a result of the low population density might be expected to cause further inbreeding depression, but low levels of competition and the relatively benign climate may have reduced the impact of selection against inbred individuals.

Low genetic variability in natural

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populations is by no means always accompanied by obvious inbreeding depression, as shown by species such as the cheetah and northern elephant seal^{7,8}. Populations that decline in numbers gradually may become inbred slowly enough to lose much of their load of deleterious mutations by selection⁹. As a result, conservation biologists have conducted a vigorous debate over the role of inbreeding depression in the extinction of species that reach low population sizes. One of the more convincing demonstrations of an increase in frequency of potentially fatal genes in a very small natural population emerged last year, with a description of the incidence of congenital heart defects and testicular non-descent in the critically

endangered Florida panther¹⁰: inbreeding depression seems to be strongly implicated in the fate of this species. But both in theory and in practice, demographic¹¹ and environmental¹² pressures may be at least as important in putting a population at risk — the important message from both the song sparrow and the white-footed mouse studies is that these three factors will rarely act independently. □

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HUMAN IMMUNODEFICIENCY VIRUS

Chaperoning a pathogen

Bryan R. Cullen and Joseph Heitman

RETROVIRUSES rely on host factors for many aspects of their replication cycle, including transcription of the integrated provirus, translation of viral messenger RNAs and post-translational modification of viral proteins. Given the known importance of cellular chaperones and enzymes for the correct folding of host proteins, it might be predicted that the appropriate folding of retroviral structural proteins, and their assembly into infectious virion particles, would also require host-protein assistance.

This prediction is now confirmed in reports by Franke *et al.*¹ and Thali *et al.*² (pages 359 and 363 of this issue) which identify, for the first time, a human protein that promotes the formation of infectious HIV-1 virions. The host factor in question is cyclophilin A, a protein whose other claim to fame is that it is the target of the clinically important immunosuppressive drug cyclosporin A (ref. 3).

Cyclophilin A is a cytosolic form of the cyclophilin family of peptidyl-prolyl *cis-trans* isomerases. Members of the family, and those of a second, distinct group of prolyl isomerases termed the FK506-binding proteins (FKBPs), are ubiquitous and abundantly expressed^{3,4}. During protein synthesis, the ribosome is thought to synthesize the peptide bond in the *trans* isomeric form. Although *trans-cis* isomerization of peptidyl-prolyl bonds can occur spontaneously, this process can be the rate-limiting step in protein folding or assembly under some circumstances. Catalysis of isomerization by the cyclophilins or FKBPs may therefore be involved in protein folding *in vivo*^{3,4}.

Cyclosporin A and FK506 exert their immunosuppressive effects by inhibiting T-cell activation. The discovery that they also specifically inhibit cyclophilin and

FKBP enzymatic activity suggested that peptidyl-prolyl isomerization might be required for T-cell activation. However, it is now known that each drug instead forms a stable complex with the relevant partner immunophilin; the complex then interacts with the protein phosphatase calcineurin to block the calcineurin-dependent nuclear import of one subunit of the transcription factor NF-AT, a critical step in T-cell activation^{5,6}.

If proline isomerization is not involved in T-cell activation, is it then essential for other cellular functions? In fact, not much is known about what the cyclophilins and FKBPs do. In unicellular eukaryotes, they are not necessary for normal cell growth, although the cyclophilins do protect somewhat against heat shock in yeast^{3,4}. In multicellular eukaryotes, cyclophilins have been implicated in folding rhodopsins in the *Drosophila* eye, and transferrin and collagen in the mammalian endoplasmic reticulum. Also, a form of cyclophilin found in endoplasmic reticulum associates with a protein involved in calcium signalling in T-cells⁷, while an FKBP has been shown to be required for proper functioning of the ryanodine receptor in skeletal muscle⁸. The new reports therefore define one of only a very few proteins that are known to require direct interaction with a prolyl isomerase in order to function.

Initial evidence suggesting that cyclophilin A interacts with HIV-1 Gag, the polypeptide precursor of the virion structural proteins, came from its identification in a screen for Gag-binding proteins using the yeast two-hybrid system⁹. This interaction was specific, in that other retroviral Gag proteins, including the Gag of the closely related simian immunodeficiency virus (SIV), proved unable to bind cyclophilin A. Nevertheless, the rele-

vance of the interaction to the replication cycle of HIV-1 *in vivo* remained unclear.

To tackle this question, Franke *et al.*¹ and Thali *et al.*² examined whether cyclophilin A was incorporated into HIV-1 virions in culture. High levels of the protein were indeed specifically packaged into HIV-1 virions but not into those of other retroviruses, including SIV. Importantly, incorporation of cyclophilin A into HIV-1 could be prevented, in a dose-dependent manner, by treatment of producer cells with cyclosporin A. Although infectivity of HIV-1 virions formed in the presence of cyclosporin A was inhibited with the same dose dependence as cyclophilin A incorporation, SIV virions (which do not package cyclophilin A) were not affected.

By constructing chimaeric Gag fusion proteins containing different segments of HIV-1 and SIV Gag, Franke *et al.*¹ show that packaging of cyclophilin A is mediated by a short segment of the capsid protein of HIV-1 containing four proline residues conserved in HIV-1 isolates. One such proline, residue 222 in HIV-1, is required for both cyclophilin A incorporation into HIV-1 virions and virion infectivity. Curiously, this proline is also conserved in SIV Gag, suggesting that it is either the context of this proline that determines its interaction with cyclophilin A or that the mutation has a more global effect on Gag function.

Overall, then, these two reports^{1,2} demonstrate that cyclophilin A is specifically incorporated into HIV-1 virion particles, and that the interaction between this protein and the HIV-1 capsid protein is essential for the formation of infectious virions. Two central questions remain. First, where does cyclophilin A act in the HIV-1 life cycle? Second, how does it exert its effect?

Although the block to incorporation of cyclophilin A does not detectably inhibit the release of HIV-1 virions, it is certainly possible that the protein may have an essential yet subtle role in virion morphogenesis. Alternatively, cyclophilin A might act during initial stages of the next viral replication cycle, for example by facilitating conformational changes in the capsid protein that are necessary for viral uncoating.

In terms of the mechanism of action of cyclophilin A, the simplest hypothesis is that it catalyses a *trans* to *cis* isomerization of a peptidyl-prolyl bond, perhaps the one preceding Gag residue Pro 222, concerned with some aspect of capsid function. The finding that an analogue of cyclosporin A which inhibits proline isomerase activity, but is not immunosuppressive, also blocks both cyclophilin A incorporation and HIV-1 infectivity is consistent with this proposal². But there are also data hinting that cyclophilin A can act as a true protein chaperone, independent of its action as a

proline isomerase¹⁰, so this hypothesis cannot be taken for granted. Indeed, the one known example of a host protein being required for virion assembly, facilitation of filamentous phage assembly in prokaryotes by the host protein thioredoxin, has proven not to require the enzymatic activity of this folding catalyst¹¹. So it remains to be seen just what role cyclophilin A plays in the HIV-1 life cycle.

An issue raised by this research is whether cyclosporin A (or, more likely, a non-immunosuppressive analogue) could be useful in the treatment of AIDS. We are inclined to be pessimistic in this regard, for two reasons. First, the levels of the drug required to prevent HIV-1 replication are also sufficient to block the enzymatic activity of the cyclophilins. Any long-term inhibition of cyclophilin-mediated proline isomerase activity would probably result in significant toxicity. Second, because the closely related SIV is not dependent on cyclophilin A for infectious virion production, it seems that HIV-1 may well be able to mutate to a cyclosporin-A resistant form. Such

mutants, if they could be obtained, might however provide insights into the role of cyclophilin A in the HIV-1 life cycle; they might also help to answer the intriguing question of why HIV-1 is apparently unique among retroviruses in requiring this particular form of host-cell assistance. □

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CATALYSIS

Fine-tuning metal clusters

Michel Boudart

MANY industrial catalysts used today are metals that are spread over the internal surface of porous materials acting as carriers or supports. Such catalysts have enormous surface areas of many square metres of metal exposed per gram of catalyst. The metal is usually in the form of clusters containing 10–100 atoms. Many, sometimes close to 100 per cent, of the metal atoms in each cluster are exposed to the reacting mixtures, and this is especially desirable in the case of expensive platinum-group metals. But as the clusters are so tiny (1–10 nm), the question arose as to the effect of cluster size on catalytic activity. Answering that question becomes hard when the clusters contain fewer than about 50 atoms, in part because of the difficulty of synthesizing clusters of uniform size with a known number of atoms per cluster. Collections of free clusters have been made in the past decade, but now, in work reported on page 346 of this issue¹, Gates and colleagues have succeeded in producing supported clusters for catalytic testing.

First, some definitions. A catalyst is a substance that brings about a chemical reaction by providing a cyclic path along which reactants become products, while the catalyst is recovered at the end of the cycle. The activity of the catalyst is expressed by the number of revolutions of the cycle per second, expressed as a turnover frequency (TOF). The latter is a

true measure of the activity of the catalyst while the number of observed turnovers before a catalyst dies is the expression of catalyst life.

The work of Gates and colleagues is the culmination of a long effort to synthesize supported metallic clusters of known identical size and structure from known organometallic clusters. The latter precursors consist of tetrahedra or octahedra of iridium atoms, denoted Ir₄ and Ir₆, respectively, stabilized as carbonyls by carbon monoxide ligands. These precursors are adsorbed in the pores of various powders (magnesium oxide, γ -alumina, sodium Y-zeolite). Then the carbon monoxide ligands are removed by heating in helium at 300 °C. The authors show by X-ray absorption that the original tetrahedra and octahedra are preserved at the end of the preparation, as well as after the catalytic reaction. The reactions chosen were the hydrogenation of cyclohexene and of toluene, because previous work had shown that their TOF did not depend on the size of the clusters. But no one knew what would happen to the TOF on clusters containing six or fewer metal atoms.

Now, when TOF values for Ir₄ and Ir₆ clusters are compared to those measured on conventional supported iridium clusters, the following conclusions are reached. First, TOF values are markedly smaller for Ir₄ and Ir₆ than for conven-

tional Ir clusters. Second, these inferior TOF values are different for Ir₄ and Ir₆, being somewhat higher for Ir₆. Third, they depend on the nature of the support, so it appears that the support interacts strongly with these tiny clusters. On the one hand, the support acts as a ligand that stabilizes their structure and prevents the clusters from coalescing; on the other hand, it is also responsible for a partial loss in catalytic activity.

In a sense, the smallest identified clusters are too small, as their TOF is less than found on larger clusters. But it should not be concluded too hastily that this milestone in a long search is disappointing. Indeed, the proof of concept is that stable, identical and identified clusters can be supported and are catalytically active. The synthetic strategy behind this result can now be exploited in two ways. First, instead of focusing on activity, a future goal might be selectivity. There is an empirical rule in catalysis according to which an increase in selectivity can sometimes be bought at the expense of activity. An example of selectivity might be the selective hydrogenation of a C=C bond in a molecule with both a C=C bond and a C=O bond, as may be desirable in the preparation of fine chemicals. Second, the next synthetic strategy might be to prepare clusters containing two different metals.

Both ideas have indeed been pursued by two collaborating groups at the University of Notre Dame, one led by Thomas Fehlner and the other by Eduardo Wolf^{2,3}. Their synthetic strategy is to prepare non-supported bimetallic porous particles of clusters by controlled thermal decomposition of single-source precursors containing both metals. These new catalysts, containing cobalt and another metal such as molybdenum, copper or zinc, have been tested for selective hydrogenation of butadiene to butene: the selectivity is higher than that observed with conventional catalysts. Another more challenging reaction, the selective hydrogenation of crotonaldehyde to crotyl alcohol, is being investigated.

In conclusion, if it is accepted that selectivity is a more important catalytic attribute than activity — as is the case for enzymes — then the synthetic strategies discussed above show promise for producing new catalysts for the rapidly growing industry in fine chemicals. □

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Exclusive receptors

Doron Lancet

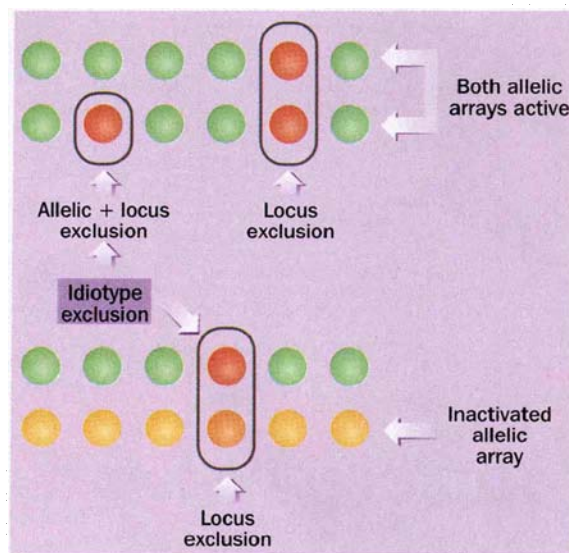
A PAPER by Chess *et al.* in *Cell*¹ provides strong indications that an old question has been answered. The question is whether the olfactory pathway obeys a one cell-one receptor rule^{2,3}, and the answer is probably 'yes'. It seems that not only does each sensory cell solely express one of the hundreds of olfactory receptor (OR) genes; but even within a given gene locus, a cell exclusively transcribes one of the two alleles ('allelic exclusion'; see figure). Such exquisite developmental fine-tuning may be an indispensable part of the process by which the brain deciphers odour messages⁴.

Olfactory sensory neurons respond to millions of chemical configurations. Each neuron has a unique, albeit broad spectrum of agonist ligands. That such spectra overlap considerably, two neurons sharing as many as 50 per cent of their stimulatory ligands, has been thought to indicate that each sensory neuron carries multiple OR types. An alternative view has it that OR proteins themselves have broad binding specificities, and that olfactory neurons each express only one OR protein type ('idiotypic exclusion'). Proponents of this second view pointed out that such clonal exclusion is often the rule in biology, citing the immune system as a prominent example². Furthermore, OR exclusion would greatly simplify the wiring and information-processing attributes at the olfactory bulb, the first chemosensory relay station in the brain^{3,4}. The newly discovered allelic exclusion of OR genes lends strong support to this reasoning.

Unravelling allelism in the olfactory system is no easy task. Faced with several hundred gene loci encoding close variants of the same protein, one would have to show that two similar sequences indeed come from a gene pair at the same locus and not from tandem loci. Chess *et al.*¹ used the well-tested system of two closely related mouse species *Mus musculus* (M) and *Mus spretus* (S) (rather than individuals or strains from the same species) to increase the probability of inter-allelic variation. They also chose the mouse OR gene I7, which has enough sequence idiosyncrasy to distinguish it from all other OR gene superfamily members (a 'single gene'). They then showed that DNA-sequence differences between the M and S forms of

the I7 gene segregated as alleles in M×S crosses.

With *bona fide* alleles identified, Chess *et al.* employed a statistics-based strategy to demonstrate allelic exclusion. Specific DNA probes revealed that small pools of dissociated chemosensory neurons from the M×S progeny that are positive for the I7 RNA, contain either the M or the S



Exclusion regimes in the olfactory system and elsewhere. Clonal exclusion is a general term denoting a state where each clonal group of cells is committed to expressing a subset (often one) out of a family of genes. Allelic exclusion is the case in which a cell expresses only one of two alleles at a given locus. Allelic exclusion at more than one gene locus in a genomic region (for example through imprinting) is called allelic inactivation (bottom). Locus exclusion is a situation in which both alleles at a given gene locus are transcribed, while loci corresponding to other members of a gene array are not (top right). This may happen if the active gene is controlled by a locus-specific *trans*-acting factor affecting both alleles equally. Idiotypic exclusion is the expression of only one gene product in a family of genes, whereby only one allele of just one locus is transcribed. This is exemplified (top left) by the transcription of only one immune receptor idiomere, whereby each of the rearranged genes coding for the functional protein shows both locus and allelic exclusion; or (bottom) by the 'cross' between locus exclusion and allelic inactivation, seen in the olfactory system. The term 'idiotypic' is applied here to olfactory receptors, in analogy with the immune system, to denote one specific functional protein, defined by the sequence of its polypeptide(s), its ligand specificity and/or its antigenic determinants.

form. Pools positive for both probes appeared only as seldom as prescribed by Poisson statistics. The inference was that only one of the two OR alleles is transcribed in any individual cell.

It remains to be seen, however, whether each cell actually transcribes an OR from only one gene locus ('locus exclusion', see figure). An alternative possibility, whereby cells express one allele from each of several gene loci, is also consistent with

the new data. Circumstantial evidence for locus exclusion does however come from previous *in situ* hybridization studies^{5,6}. Many OR probes were found to label 1/200–1/500 of the cells in olfactory epithelium, with only rare doubly labelled cells. With current estimates for the number of OR gene loci ($N_{\text{orf}} = 200\text{--}1,000$), a labelling frequency of about $1/N_{\text{orf}}$ is consistent with (but does not prove) locus exclusion^{1,7}. More direct evidence could come from applying reverse transcription and the polymerase chain reaction to single sensory cells, with the aim of showing that only one OR transcript is present. But in this approach, the enormous noise due to potential co-amplification of hundreds of homologous genomic DNA receptor copies would have to be carefully kept at bay.

The immune system achieves idiomere exclusion through DNA rearrangements that, by a cunning inhibitory feedback, can happen only once in any cell. So when one allele at one locus becomes competent for transcription, no other can do so, resulting in locus exclusion and allelic exclusion in one go⁸. A different mechanism may operate in the olfactory system.

Using elegant fluorescent imaging of interphase DNA replication asynchrony, and based on the known correlation between replication time and allelic inactivation, Chess *et al.* suggest that one of the two allelic copies of an entire cluster of OR genes may be inactivated early in embryogenesis. It is then necessary to explain locus exclusion by additional gadgetry, which may involve *cis*- and *trans*-acting regulatory devices, including those that allow each OR locus to be expressed only in one of several anatomical zones of olfactory epithelium^{1,5,9}.

But why should the olfactory system go to the effort of attaining both locus exclusion and allele inactivation? For many other genes, expressing both alleles at a given locus may be selectively advantageous (if one allele is defective) or neutral (if both alleles are functionally identical). Could

the expression of both alleles be deleterious in the olfactory pathway? This would be true, given two conditions⁷: first, that one and only one OR specificity must appear on the surface of a given chemosensory neuron, to convey a well-defined neuronal message to the brain; second, that functional heterozygosity is prevalent in olfaction (that is, allelic OR gene products often have different odorant specificity). This second condition

implies a genetic polymorphism, which could be driven by the evolutionary regime known as balancing or overdominant selection¹⁰.

A well-fathomed example is the genes of the major histocompatibility complex, where the population harbours numerous, functionally distinct alleles at each locus¹⁰. The result is a large degree of population diversity in the immune response, crucial for the survival of the species. In the olfactory system, functional heterozygosity could potentially double the number of different odorant binding sites encoded in the genome, and so would be important for the olfactory acuity of the individual⁷. That several functionally distinct alleles may exist at some OR gene loci is consistent with the considerable individual genetic variation in human olfactory thresholds for certain odorants¹¹.

It is likely that the olfactory system first evolved a relatively small number of OR genes, together with the mechanism of locus exclusion. This is attested to by the observation that even the 'lower' vertebrates seem to obey the $1/N_{\text{olf}}$ labelling law⁶. The selection rule may then have become 'the more ORs the better', so that

alleles often began to encode ORs with different specificities. It was then that allelic inactivation became necessary. The olfactory system may thus have recently borrowed a mechanism that elsewhere (for example in X-chromosome inactivation¹²) appeared very early in evolution. How such curious adaptation may have occurred is an issue for the future. □

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CLIMATE CHANGE

Dual effects of ozone reduction

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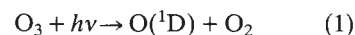
FOR years ozone depletion and climate change have been considered separate problems, with little or no overlap. A different and more complex picture is now gradually emerging as our understanding of climate–chemistry interactions increases. Atmospheric chemistry can affect the climatically important gases in many ways; for instance, the latest international assessment¹ suggests that the climate impact of methane could be twice as large as previous estimates because of the indirect effects of atmospheric chemistry, whereas the indirect (radiative) effect of chlorofluorocarbons, through their reduction of stratospheric ozone, is similar on a global scale to their direct effect, but of opposite sign². So methane becomes a highly effective greenhouse gas, whereas the chlorofluorocarbons probably are negligible. On page 348 of this issue, Touni and co-workers³ now shake up our ideas on stratospheric ozone.

The point raised in their paper is that reductions in ozone, which will increase the flux of ultraviolet radiation reaching the lower atmosphere, will lead to increased formation of sulphate cloud condensation nuclei (CCN), and so to increased numbers of cloud droplets. The result will be to reduce solar heating of the lower atmosphere, thereby acting as a negative forcing effect. As clouds are

known to play an important role in the Earth's energy balance by reflecting a substantial fraction of solar radiation, even a small change in this reflection due to increased droplet levels could be significant compared with radiative changes due to changes in the greenhouse gases. In fact, it is possible that this negative indirect effect could be similar to other identified indirect effects.

Research into the role of sulphate aerosols for the radiative balance of the Earth has become a high priority over the past few years. Wigley⁴ suggested that temperature increases in the Northern Hemisphere could have slowed down because of increased CCN formation from anthropogenic emissions, and there are recent studies¹ indicating that this is the case. Charlson *et al.*⁵ suggested that non-cloud sulphur aerosols, mainly from anthropogenic emissions, have a similar substantial impact on climate. These studies deal first and foremost with sulphate pumped into the atmosphere by anthropogenic emissions of sulphur dioxide. What is new in the research by Touni *et al.*³ is that it directly links changes in the ozone layer to the climate effect of clouds. It thereby adds to the other negative climate effects believed to have resulted from increased emission of sulphur over the past few decades.

Ozone layer depletion has further implications for climate. The key point is that reduction in stratospheric ozone will lead to enhanced penetration of ultraviolet-B radiation into the troposphere where ozone will be dissociated. In turn, this will lead to hydroxyl radical (OH[•]) formation through the reaction sequence



Reaction (2) is the main source of hydroxyl radicals in the free troposphere, and OH[•] is a key compound in the tropospheric oxidation process. So this reaction controls the fate of large numbers of climatically active chemical compounds. Studies^{6,7} that have dealt with the interaction between ultraviolet-B changes and changes in oxidation in the troposphere, affecting greenhouse gases such as O₃ and CH₄, seem to indicate that increases in ultraviolet-B radiation reduce the global distribution of O₃ and CH₄, thus giving a negative contribution to the radiative forcing⁸.

Tropospheric OH[•] formation is sensitive to the thickness of the ozone layer. Therefore, the marked reductions in global ozone that have been observed through the 1980s, and particularly the early years of the 1990s, are likely to have led to more OH[•] formation. For instance, observations by the total ozone mapping spectrometer on the Nimbus 7 satellite suggest that, worldwide, total ozone has fallen by close to 4 per cent over the past 15 years¹, indicating a significant increase in the short-wave solar radiation leading to OH[•] formation. One of the key questions is how well the observed ozone changes can be connected to human activity. Although there are gaps in our understanding of the atmospheric behaviour of ozone, and the analysis is complicated by the influence of natural events such as the volcanic eruption of Mount Pinatubo, there is increasing evidence that the long-term reductions observed in the Northern Hemisphere over the past two decades⁹ stem from increased anthropogenic emissions of chemicals.

The significance of the hydroxyl radical for the formation of CCNs and thereby for climate lies in the OH[•] oxidation of SO₂

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to sulphate, the main source of new sulphate particles in the troposphere. But the links in the chain of events from OH[•] changes via sulphate particle formation to the impact on the Earth's radiation balance remain shaky, because we do not yet know enough about the intermediate processes. Although sulphate particles are important as CCNs, there are other compounds that contribute (organics, mineral dust, sea salt) to varying extents in different regions. Formation of sulphate through the reaction chain above is likely to modify the size distribution of droplets, and this will have an impact on the optical properties. The liquid water contents of clouds and lifetime of droplets are also likely to change. Nevertheless, the limited studies conducted so far suggest that the impact of

sulphur via CCN formation is negative, and probably of the same order as the direct cooling effect of sulphate-particle reflection of solar radiation.

As the work of Toumi and co-workers illustrates, the introduction of indirect radiative effects acting through chemical changes in the atmosphere increases the complexity of the issue, with no clear linear relation between emissions and changes of the climate component. Nevertheless, such processes are evidently of potential significance. We will have to grit our teeth and include them, in all their complexity, in future studies of greenhouse gases. □

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IMMUNOLOGY

New tricks for old molecules

Jeffrey V. Ravetch and David H. Margulies

In its protected environment, the fetus receives antibodies from its mother so as to prepare it for the rigours of the outside world; after birth, further consignments are delivered in the maternal milk. Some of the molecular mechanisms that underlie the transfer are now revealed in papers on pages 336¹ and 379² of this issue. The papers come from Pamela Bjorkman's group. They describe the high-resolution, three-dimensional, X-ray crystallographic analyses of a rat neonatal antibody receptor, FcRn, and its complex with the Fc portion of immunoglobulin G (IgG). They reveal the first structure of an Fc receptor and a surprising application for a protein fold employed by molecules of the major histocompatibility complex (MHC); moreover, together with a study³ identifying its human placental counterpart, hFcRn, they offer insights into the mechanisms of Fc binding and IgG transport.

The Fc receptors (FcR) are a diverse family of membrane glycoproteins that bind the carboxy-terminal part of the constant region of immunoglobulins, function as effector molecules and direct the transport of immunoglobulins across epithelial cells. On most haematopoietic cells, FcRs are responsible for a wide variety of cellular responses to antibodies, including IgE-mediated allergic reactions and IgG-triggered cytotoxicity and inflammation, the result of crosslinking cognate Fc receptors by antibodies⁴. This group of FcRs are structurally related, hetero-oligomeric molecules in which the ligand-binding subunit is composed of tandemly repeated immunoglobulin-like domains.

In contrast, the transport of maternal IgG to the fetus and newborn is mediated

by the heterodimeric FcRn, whose heavy chain is similar in sequence to that of the heavy chain of MHC class I molecules. This receptor assembles with the same light chain used by MHC class I, β_2 -microglobulin (β_2m)⁵.

In the suckling mouse and rat, maternal

IgG binds to the FcRn expressed on the intestinal epithelium at the acidic pH of ingested milk, and the FcRn-IgG complex transcytoses to the serosal surface where the maternal IgG dissociates at the neutral pH of the blood⁶. Transport of IgG across the fetal yolk sac involves the same FcRn molecules⁷ with a similar pH dependence of binding. However, because there is no pH gradient across the maternal-fetal interface, IgG binding to FcRn is unlikely to be occurring at the cell surface; rather, yolk sac FcRn is localized in apical vesicles where it encounters IgG taken up by fluid-phase endocytosis. Binding of IgG to FcRn in these acidified endocytic vesicles occurs, followed by the dissociation of the complex on delivery to the blood. A similar scheme for transport of human placental IgG is supported by the identification of an FcRn homologous to the rodent receptors³.

Earlier studies had identified receptors of another class, Fc γ RII and III on the placental endothelium and syncytiotrophoblast, respectively, and it was proposed that they are involved in placental IgG transport. But these low-affinity receptors are unlikely to be significant in immunoglobulin transport. Fc γ RII and III preferentially engage immune complexes, triggering cellular responses through pathways mediated by tyrosine kinases. They do not bind monomeric IgG and exhibit no pH dependence in their association with IgG.

The similarities in the amino-acid sequences of MHC class I molecules and FcRn raise the question of whether they have any similarity of function. The MHC class I molecule samples peptide fragments from the endoplasmic reticulum and displays a subset of them at the cell surface for specific recognition by T lymphocytes bearing $\alpha\beta$ T-cell receptors. The MHC class I structure has a remarkable groove, formed by the antiparallel placement of two α -helical regions upon a table of β -sheet. This groove binds self- or antigenic peptides by accommodating their termini and amino-acid side chains in complementary pockets. The stable heterotrimeric complex (class I molecule/peptide/ β_2m) interacts with the T-cell receptor as well as the coreceptor molecule CD8. So the class I molecule has evolved for tight interaction with some peptides and molecules with immunoglobulin-like domains.

The three-dimensional crystallographic structure of FcRn reveals striking similarity in its backbone fold to that of MHC class I molecules. In particular, the α_1 and α_2 domains of the FcRn show a very similar pattern of helices supported by β -strands (Figs 1a and b). However, the helices are not separated, and the interaction of their side chains obliterates the potential groove. This is consistent with the observations that FcRn, unlike almost

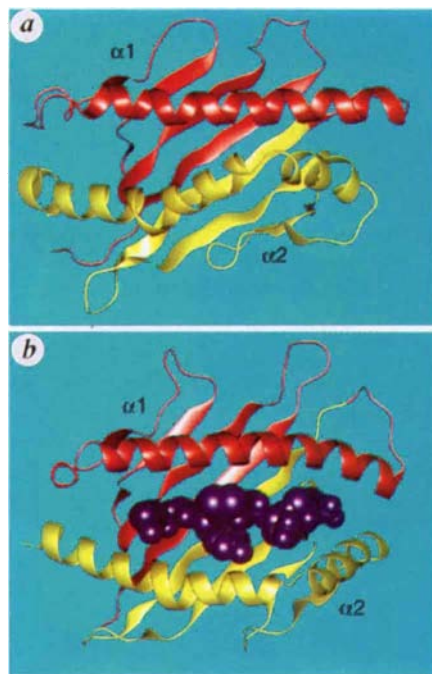


FIG. 1 Comparison of the α_1 and α_2 domains of FcRn (a) with those of an MHC class I molecule (b). These ribbon diagrams show the α_1 and α_2 domains of the FcRn in red and yellow, respectively, and the murine MHC class I molecule, H-2K^b, complexed with an antigenic peptide (purple) derived from the vesicular stomatitis virus¹⁰. (Details taken from ref. 1.)

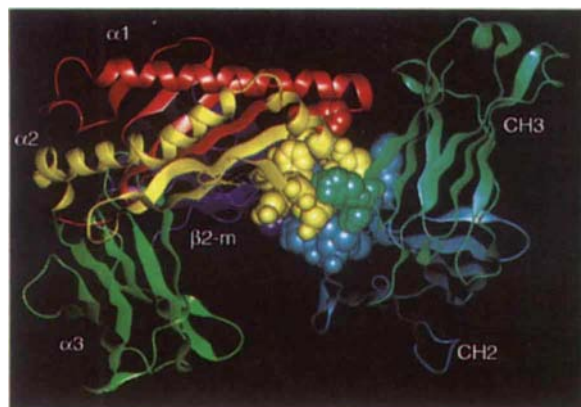


FIG. 2 Principal interactions between the FcRn and Fc, illustrated by ribbon diagrams of FcRn (domains $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 2m$ are in red, yellow, green and purple, respectively) and of Fc (CH2 and CH3 domains in blue and light green). The main contact residues ($\alpha 1$ domain, 90; $\alpha 2$, 113–119 and 131–135; $\beta 2m$, 1–4 and 86) are depicted as space-filling structures. Contact residues from $\alpha 3$ FcRn dimer to Fc are not shown. (Details taken from ref. 2.)

all MHC molecules studied, does not copurify with bound peptide.

The structure of the co-crystal of FcRn complexed with Fc, at pH 6.5, reveals the main site of contact between the two molecules. This is at the edge of the $\alpha 1/\alpha 2$ domain region, a region distinct from the sites of interaction with peptide, CD8 or T-cell receptor. This part of the Fc-binding site of the FcRn (Fig. 2) is formed by three β -turns of $\alpha 1/\alpha 2$, lying to the side where the carboxy-terminal portion of bound peptide resides in a classic class I molecule. Additional contributions to Fc binding derive from the amino terminus of $\beta 2m$ and a loop from that domain. The surface of the Fc that interacts with the FcRn derives from both the CH2 and CH3 domains, and probably exploits three or four titratable histidines as contacts. Both the isolated FcRn and the complex with Fc reveal higher order dimers, formed by juxtaposition of the Ig-like domains of the FcRn. The reason for such dimerization is unknown, but does offer a compelling model for the interaction with IgG.

So a new functional role has been adopted by using yet another surface of this versatile molecule. With the available evolutionary comparisons, it is impossible to predict if the common ancestor of MHC and FcRn had a functional groove which was lost by FcRn after divergence of the molecules, or if MHC molecules acquired a functional groove driven by their need to display self- or antigenic peptides for presentation to T cells. Further evolutionary comparisons certainly seem warranted. Moreover, although both structural and biochemical data strongly support the notion that this MHC-like molecule does not employ its potential groove to bind peptide, the question remains whether this site might interact with other ligands. The demonstration⁶ that CD1b, a non-polymorphic, $\beta 2m$ -associated mem-

ber of the MHC family binds a lipid antigen, mycolic acid, and presents it to T cells with $\alpha\beta$ T-cell receptors, raises the possibility that FcRn may also play a role in presentation of non-peptide antigens.

How general might this first, three-dimensional structure of an Fc receptor be? Two lines of evidence suggest that it may well tell us something about other FcRs of the immunoglobulin domain family. First, modelling of the IgE high-affinity FcR, Fc ϵ RI — based on deletion studies, sequence comparisons with CD4 and CD2, and estimates of the distance from the amino to the carboxy terminus of IgE — indicate

a 'lying down' structure, where the long axis of the molecule sits parallel to the membrane⁹, allowing a dimer interaction similar to that observed in the FcRn structure. Second, the greatest sequence similarities between FcRn and other FcRs lie in the $\alpha 3$, Ig-like domain, which may contact the CH2 domain in the dimer predicted by this model. The predictive value of such modelling will be readily testable by mutational mapping of potential IgG-binding sites.

Each unique member of the Fc receptor family has attracted considerable attention for its importance in mediating various aspects of the immune response, ranging from lymphocyte modulation to inflammation and immunoglobulin transport. Given the surprises to be found in the three new papers^{1–3}, further three-dimensional structures of each of these molecules, and of their complexes with Fc, will be eagerly awaited. □

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Free the spirit!

LAST week, Daedalus was expounding the physics of poltergeists. The spirit world, he reckoned, coexists in the same space as the material one, but is much colder: perhaps at the cosmic background 3 K. A poltergeist works by abstracting radiant heat from the material world and letting it run down into the spiritual world. It is simply a heat engine.

He is now risking a more teleological interpretation. The common theory of ghosts, he points out, is that they are the souls of dead people, disconsolately haunting the region where they died. Only a few unlucky souls seem to get stuck in this way. Possibly such a soul fails to carry away sufficient animal heat from its previous abode: in the cold spiritual world it is simply frozen to the spot. To free itself for further adventures, it needs to acquire enough latent heat of evaporation. Haunting is simply its attempt to steal heat from the material world. This is why it causes cooling. The disturbance and noise may be pure waste motion, a sort of counterpart to waste heat. Alternatively, they may be intended to stir up helpful emotions in the human audience. A human mind is, after all, a link between the two worlds, and may be essential to the heat transfer process.

This theory has several implications. It suggests that cremation, which gives the soul the heat it needs to fly free from the body, is the best way of disposing of the dead. This may have been sensed by those ancient cultures who burnt their nobles on heroic funeral pyres. It implies that a dying individual should undergo a sharp drop in temperature, as his departing soul leaves for the spirit world and takes a little heat with it. It further suggests that, in the traditional exorcism ritual with bell, book and candle, it is the candle that does most of the work. It provides the necessary radiant energy.

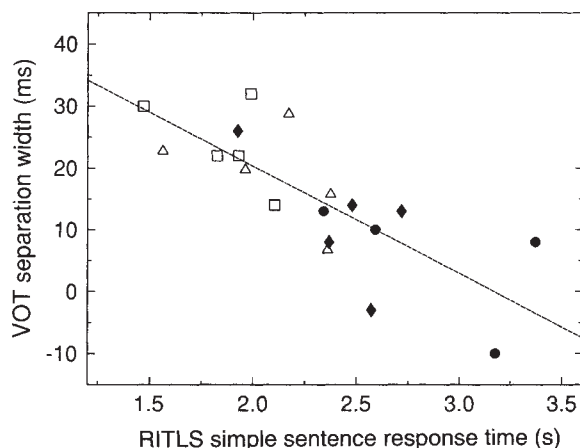
But radiant energy from a candle flame at some 2,000 K is badly matched to a sink at 3 K. A truly efficient exorcism would warm the frozen ghost with matching 3 K 'cosmic background' microwave energy. The radiation from a standard microwave oven would probably do very well. Furthermore, its hundreds of watts of power would be far more effective than any candle. At first Daedalus feared that the oven would need to be rigged to work with its door open, to flood the room with microwaves. But he now reckons that the ghost could pass through the oven wall to access the inside directly. Bell, book and microwave oven would free the trapped ghost to roam the spirit world, and free its human hosts from its haunting, chilling pleas for release.

David Jones

Cognitive defects at altitude

SIR — Hypoxia, occurring at high altitude, is known to cause decrements in normal neural functioning¹. We propose a method to assess such effects remotely in real time from naturally occurring speech. We studied the effects of extreme altitude on the neural processes that underlie speech motor control, syntactic comprehension and other cognitive functions in five male members of the 1993 American Sagarmatha expedition to Mount Everest.

We measured speech 'voice onset timing' (VOT), which differentiates English 'voiced stop' consonants (/b/, /d/ and /g/) from their unvoiced counterparts (/p/, /t/ and /k/, respectively) in word-initial position. Syntax testing was done with the Rhode Island test of language structure² (RITLS). The confrontation naming task, forward and backward digit-span, and the odd-man-out tests from the DATATOP series³ were also administered at each location to assess memory, attention, and maintenance and shifting of cognitive sets.



VOT separation width plotted against reaction time to the simple items of the RITLS for five subjects at four locations (one point missing due to a recording error). The correlation coefficient and the linear regression equation are also shown. Subjects were tested at base camp (altitude 5,300 m) before (□) and after (△) a summit climb attempt; at camp two (6,300 m; ◆), and at camp three (7,150 m; ●), within a day of arriving at each location. No supplementary oxygen was used at the testing altitudes. Each subject read on each location 60 monosyllabic English words that had voiced and unvoiced stop consonants in initial position. A recording was made through VHF radios using micro DAT recorders to ensure an accurate time basis for the measurement of VOT—of the time between the onset of the burst (produced by lip opening or tongue lowering) and the onset of phonation (produced by vocal fold vibration). For each subject on each location, the VOT separation width was defined as the time interval between the longest voiced and the shortest unvoiced VOT for all three places of articulation combined. The RITLS was administered by showing the subject a page (for each sentence) presenting three line drawings, one of which best exemplified the meaning of the sentence which was then read aloud by the experimenter. The test sentences and the subjects' responses were recorded through VHF radios; response time was determined by measuring the time interval between the end of each spoken sentence, and the subject's announcing the number of the matching sketch. A different 50-item balanced version of the RITLS was used on each location.

VOT separation width (the distance in time units between the longest voiced and the shortest unvoiced VOT), decreased significantly at higher altitudes ($F(3,12) = 6.30$, $P = 0.008$). The response time to the 'simple' RITLS items increased significantly at higher altitudes ($F(3,12) = 14.69$, $P < 0.0005$) and was negatively correlated with VOT separation width ($r = -0.774$, $P = 0.0001$). The figure shows the linear relation between the two types of measurements.

Response times to the complex items of the RITLS were always longer than those to the simple items ($F(1,4) = 30.56$, $P = 0.005$), so response times can be assumed to reflect processing difficulty. The cognitive slowing down exemplified by longer response times to the simple items was not general because the number of words produced in the confrontation naming task (a timed task) was not affected by altitude ($F(3,12) = 1.14$, $P = 0.373$), and neither was the response

time to the complex items ($F(3,12) < 1$). The subjects were not cold or uncomfortable at the high camps. Fatigue could not have caused the differences because the final testing at base camp was done after the longest and most strenuous part of the climb (including climbing up to the summit and back down to camp two and to base camp within one day), yet subjects' VOT separation width was longer and syntax response time shorter than at the high camps. Fatigue also does explain the differential deficits we found.

Similar patterns of deficits are found in patients of Parkinson's disease⁴ and may be the result of disruption of subcortical basal ganglia pathways to prefrontal cortex⁵. Mild anoxia of subcortical circuits to prefrontal cortex seems to be a plausible explanation of our findings, as basal ganglia are known to be sensitive to hypoxic insults⁶. Whatever the extent of the cognitive deficits turns out to be, it is very important that simple speech measurements may serve to assess them remotely in real time. Further study is necessary to determine the relation-

ships between cognition and speech motor control in other situations and to develop and validate a compact, automatic speech analysis system for remote monitoring. Such systems can be very useful in situations where critical crew behaviour (for example, in aeronautics, space flight or air-traffic control) may be endangered by hypoxia, high carbon-monoxide levels, or drug or alcohol intoxication.

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Interatomic signalling in QED

SIR — In his article¹ "Time machines still over the horizon", Maddox writes misleadingly that vacuum polarization is a phenomenon "for which Feynman, Schwinger and Tomonaga, with the help of apologists such as Dyson, were responsible late in the 1940s". In fact the idea of vacuum polarization is contained in much earlier papers of Dirac and Heisenberg, and quantitative results for the modification of the Coulomb potential by the polarization of the vacuum were derived by Serber² and Uehling³ in 1935. Feynman and Schwinger rederived these results and used them in the calculation of radiative corrections.

Maddox also states that Fermi's work⁴ on his two-atom model of light propagation in quantum electrodynamics (QED) was carried out at the suggestion of Heisenberg, when in fact it was Kikuchi⁵, not Fermi, to whom Heisenberg suggested the problem; and it was Kikuchi, not Fermi, who "first tackled the problem".

But the most serious error is the claim that "a sixty-year old calculation by Enrico Fermi is discovered to be in error". In his calculation of the probability for an initially excited atom to excite a second atom a distance R away, Fermi replaces an integral involving all (positive) photon frequencies ω by one in which the integration is extended to all negative photon fre-

quencies. This procedure is necessary to demonstrate causal propagation in the so-called rotating-wave approximation made by Fermi. But when this approximation is not made and one works with the full electromagnetic field, properly retarded signalling is no more difficult to demonstrate in QED than in classical electrodynamics⁶. This result is hardly surprising, as the Maxwell equations for the Heisenberg-picture field operators have the same formal structure as in classical electrodynamics⁷. One obtains exactly the results of myself and Knight^{8,9} and therefore, as a special case, the result of Fermi¹⁰. Regarding Hegerfeldt's theorem¹¹ which "shows there is a small probability that atom B will 'notice' the decay of A long before the interval R/c has elapsed"¹, it should be noted that the theorem as stated does not even require the presence of a second atom. Its relevance to interatomic signalling seems dubious at best. Fermi's calculation, far from being "in error", exemplifies his inimitable, direct and pragmatic style.

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Ape family tree

SIR — The recently discovered *Dryopithecus* partial cranium CLL-18000 is reported to have several derived features of the zygomatic bone¹, including three zygomaxillary facial foramina on its frontal process, which establish this taxon as a primitive member of a *Pongo* clade excluding African apes and humans¹. But crania of extant hominoid primates are highly variable² and polymorphic for zygomaxillary facial foramina numbers (table). In a Liberian chimpanzee cranial sample, the character state of three foramina per side in any combination, unilaterally or bilaterally, occurs in 22.8% of the specimens, approximately one in four.

These findings are reinforced by a survey of Asian hominoids: *Pongo pygmaeus*, *Symphalangus syndactylus*, and *Hylobates* spp. Phenotypes differ in various complex ways but a few patterns are clear. *Pongo* is so highly variable for this epigenetic feature that its numerical range encompasses

FREQUENCY DISTRIBUTION OF ZYGOMAXILLARY FORAMINA IN EXTANT HOMINOIDEA

Taxon	Numbers of foramina (right side)								
	N	1	2	3	4	5	6	7	8
<i>Pan troglodytes</i>	250	87	138	24	1	0	0	0	0
<i>Pongo pygmaeus</i>	89	9	17	25	19	12	4	1	2
<i>Symphalangus syndactylus</i>	11	2	6	3	0	0	0	0	0
<i>Hylobates muelleri funereus</i>	19	9	6	3	1	0	0	0	0
<i>H. lar entelloides</i>	20	0	9	5	3	2	1	0	0
<i>H. lar carpenteri</i>	17	0	3	8	3	3	0	0	0

All observations comprise specimens collected in the wild, with Liberian *Pan troglodytes* curated at the Frankfurt Anthropological Institute and Asian hominoids curated in the Department of Mammology at the US. National Museum of Natural History (Smithsonian). Numbers of zygomaxillary facial foramina are given for the right side only, corresponding to the preserved region of CLL-18000.

all other extant hominoid genera as well as the CLL-18000 specimen attributed to *Dryopithecus*. However, even with the wide range of variation subdivided into just two character states, *Pongo* exhibits a lower percentage of specimens with three or more foramina than does at least one taxon, *Hylobates lar carpenteri*, in a reportedly plesiomorphous genus¹. Because all extant hominoid taxa are polymorphic for zygomaxillary facial foramina, the number of foramina in any individual, extant or fossil, is unreliable as an indicator of phylogenetic relationship. Taken alone, the numbers of zygomaxillary facial foramina in CLL-18000 do not provide support for removing *Dryopithecus* from a position ancestral to extant African Hominoidea and humans, because the fact that a specimen has two or three zygomaxillary facial foramina does not necessarily indicate whether it is primitive or derived. Instead, the character state present in ancestral hominoids was probably the same as that encountered in all extant taxa closely related to them: a polymorphism of considerable phenotypic complexity.

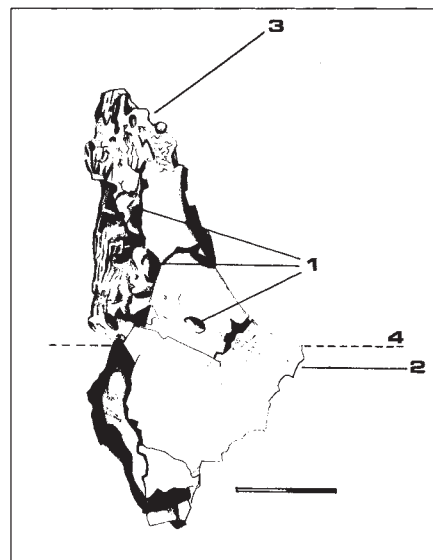
Assignment of CLL-18000 to a *Pongo* clade was also based on reported robustness of the specimen's zygomatic bone as well as the location of foramina relative to the orbital margin¹; subsequent work³ adds low position of the frontozygomatic suture and low position of the glenoid fossa relative to the external auditory meatus to the characters reportedly shared by CLL-18000 and *Pongo*. When these features are illustrated and described in sufficient detail, and shown to be shared with other *Dryopithecus* specimens, it should be possible to structure further tests of hypotheses concerning the phyletic position of the intriguing Can Llobateres hominoid material.

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MOYÀ-SOLÀ AND KÖHLER REPLY — We fully accept the principle that variability renders certain morphological characters inappropriate for assessment of phylogenetic relationships of isolated specimens. Our hypothesis that *Dryopithecus* is a primitive member of the *Pongo* clade would certainly be questionable if based exclusively on numbers of zygomaxillary facial foramina, which do indeed show some variation.

In fact, we referred in our paper¹ to a combination of characters, observed only in *Pongo* among extant hominoids. The zygomatic of *Pongo* is clearly distinguished from those of African apes and hylobatids by a unique association of morphological characters: elevated average number of foramina (typically three); foramina situated above the inferior orbital rim and relatively close to the frontozygomatic suture; zygomatic very broad and robust; and zygomatic flat and anteriorly oriented.

As reported in our paper¹, the zygomatic of CLL-18000 shows the same com-



Right zygomatic of *D. laietanus* (CLL-18000) from Can Llobateres (Spain). 1, Zygomatic foramina; 2, zygomaxillary suture; 3, frontozygomatic suture; 4, lower limit of the orbit. Scale bar, 1 cm.

bination of characters as *Pongo*. The uniqueness of this combination leads us to interpret this morphological pattern as a synapomorphy shared by the two taxa.

By contrast, the combination of characters observable on the zygomatic of *Hyllobates lar carpenteri*, despite the somewhat elevated number of zygomaxillary facial foramina, differs from that of *Pongo* in the following features: foramina situated at or below the inferior orbital rim, far from the frontozygomatic suture; very slender zygomatic; and convex surface of the zygomatic. The morphology of zygomatics of African apes is comparable to that of *Hyllobates*, but they generally have fewer zygomaxillary facial foramina.

We have now added two new characters observable on both, CLL-18000 and *Pongo* crania: low position of the frontozygomatic suture; and low position of the glenoid fossa relative to the external auditory meatus. Both these characters strengthen the hypothesis that *Dryopithecus* is linked to the *Pongo* clade¹ and reinforce the idea that the zygomatics of *Pongo* and *Dryopithecus* are not fortuitously similar. Further confirmation is provided by the consistent presence of a similar combination of characters in another facial specimen of *Dryopithecus*^{3,4}.

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Health risks of energy saving

SIR — The use of reduced ventilation for domestic energy conservation (draught proofing or double glazing) increases the household's radiation dose from radon gas. The health impact of this in an average home in the United Kingdom is at least 10–100 times greater than the health impact of electricity production and supply.

Scientific discussions on energy policy have focused on comparing different means of energy supply. Conservation seems to be universally viewed as having no risks attached; indeed, conservation is advocated indiscriminately by many pressure groups. Two domestic energy conservation measures, draught proofing and double glazing, deserve special consideration because they work by reducing ventilation. This reduction produces an increase in the radon level in the building's atmosphere, resulting in an increased radiation dose to the occupants.

The energy savings produced by draught proofing or double glazing¹ and the increase in radon level produced by these same measures² have been discussed previously. The health 'cost' of the increase in radiation dose is simply:

$$\text{Cost} = D \times P \times O \times C \times V/E \sim 7 \text{ pence per kWh}$$

Where D = average person's dose due to radon in the home (1.1 millisievert per year); P = fractional increase in radon level due to reduced ventilation (0.3; from ref. 2); O = number of individuals per average household (2.4); C = probability of dying from a radiation-related cancer (0.05 per sievert; ref. 3); V = value of a statistical life (£2 million; ref. 4); and E = energy conserved by draught proofing or double glazing (1,200 kWh per year; ref. 1).

HEALTH COSTS OF PRIMARY ENERGY SOURCES

External health cost adder (pence per kWh)	Source of primary energy
0.01–0.1	Supply of electricity by nuclear, solar, wind, hydro or gas
0.01–0.1	Supply of electricity by coal or oil
1–10	Energy conservation by draught proofing or double glazing

Evaluating the health impact as a cost allows it to be compared directly with the health impact of means of energy supply. Such a cost is usually described as an external adder as it is not included in the prevailing market cost and should be added to it to reflect the 'true' overall cost. In high radon areas, the health risk posed by this gas in individual properties could be up to about 100 times greater than the average.

The health impacts of electricity production have been evaluated in, for example, refs 4 and 5, and the results are summarized in the table. It can be seen that the use of reduced ventilation for domestic energy conservation has a health cost 10–100 times greater than the health cost of supplying primary energy to the domestic consumer.

Those faced with making choices between energy policy options, or involved in giving advice to the public, need to be aware that at least one generic

form of energy conservation carries a very significantly larger human health risk than means of supplying energy. The public needs to keep the risks posed by, say, the radioactive discharges from nuclear power stations in perspective — draught proofing and double glazing present a much greater threat to human health.

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Acid water and fish death

SIR — Acidification of rivers and lakes has caused decline in fish stocks in many areas affected by man-made emissions of sulphur dioxide and nitrogen oxides¹. Deaths of fish typically occur in acid 'episodes', when there is high runoff from the surface of acid soils. In addition to the direct atmospheric input, ion-exchange processes and accumulated sulphate and nitrate in the soils partly cause the acidity and toxicity of the runoff water.

Here we report on a major episode of fish deaths in southern Norway. On this occasion, the acid surge was caused by very high deposition of sea salts, resulting in the release of hydrogen and aluminium ions from the soil by ion exchange. To our knowledge, this is the first reported incidence of large-scale episodic acidification and fish deaths due to the sea-salt effect. Such sea-salt episodes cause short-term acidification of watercourses in coastal regions both in Norway² and the eastern United States³. The effect has also been demonstrated by artificial dosage of dilute sea water to a small catchment⁴.

The sea-salt effect⁵ is a direct ion-exchange process, in which Na^+ ions in the percolating water exchange with H^+ , Al^{3+} and base cations. Cl^- acts as a mobile anion and passes through the soil in relatively unaltered concentration. The acidification of the runoff will be noticeable only if either there are large amounts of precipitation with much higher concentrations of sea salts than usual, or there is large dry deposition of sea salt followed by rain. Because the release of H^+ and other cations from the soil is related to Na^+ retention, the concentration of Na relative to Cl provides a measure of the acidification potential caused by the sea-salt effect.

From 4 to 24 January 1993, an extremely low pressure over the North Atlantic gave strong southwesterly winds and large amounts of precipitation in southwestern Norway. Wind speeds at the coast were typically between 10 and 25 m s^{-1} , and daily precipitation a few kilometres inland were 10–70 mm, resulting in monthly precipitation of 200–700 mm. (annual precipitation is 1,500–3,500 mm).

1. UK Department of Energy Efficiency Office, *Read How Helping the Earth Begins at Home*, **93 EP 702** (October 1993).

2. Wrixon, A. D. et al. *Natural Radiation Exposure in UK Dwellings* NRPB-R190 (1988).

3. *Recommendations of the International Commission on Radiological Protection* ICRP Publ. 60, (1991).

4. Pearce, D., Bann, C. & Georgiou, S. *The Social Cost of Fuel Cycles* CSERGE Report to the UK Department of Trade and Industry (1992).

5. Ball, D. J., Roberts, L. E. J. & Simpson, A. C. D. *An Analysis of Electricity Generation Health Risks — A United Kingdom Perspective* CERM Research Report No. 20 (1994).

Chemical analyses of precipitation showed Cl^- concentrations of 10–50 mg l^{-1} , corresponding to a total monthly input of Cl^- in precipitation of 10–20 g m^{-2} (see figure for locations). Although concentrations of sea-salt components were three times higher than the yearly average values, other components, such as non-marine sulphate, nitrate, ammonium and hydrogen, were well below the annual mean concentrations. Concentrations of airborne sea-salt aerosols were also high. The sea-salt concentrations in precipitation were the highest recorded since monitoring started in 1973.

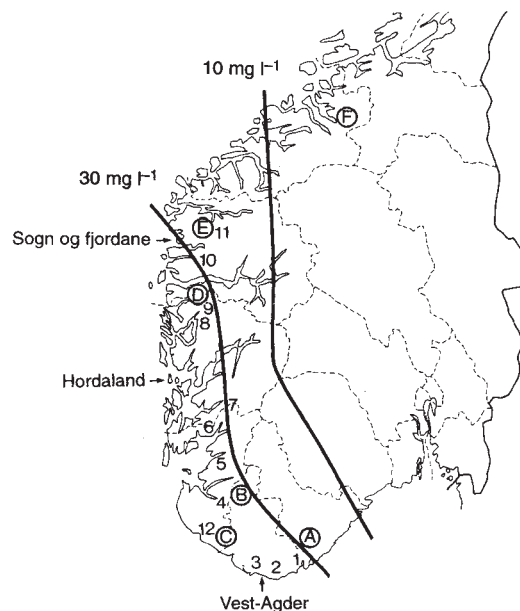
The rivers to the west of River Tovdal (river 1 in the figure) had elevated Cl^- concentrations after the storms (table). The sea-salt effect was reflected in a 'negative' non-marine Na concentration. The relative Cl^- increase was significantly correlated to the Na deficit ($r^2 = 0.81$; $n = 15$). The relative increase was highest (ratio close to 5) in rivers 9–11 on the west coast of Norway. Deaths of Atlantic salmon were reported in the River Ekso (no. 9). Sea-salt deposition did not occur in rivers east of River Tovdal.

The change in the concentration of Na^+ relative to Cl^- must be compensated by a change in other components in the water to maintain ion balance. Acidified catchments apparently responded by increases in H^+ and Al^{3+} (rivers 1, 2, 3 and 6, and three small streams in the catchment of River Bjerkreim; see table). Slightly and moderately acidified catchments responded by increases in base cations or by base cations together with H^+ and Al^{3+} (the remaining rivers, see

table). The extreme effect of the storm on the afforested catchment Svela in the River Bjerkreim catchment (stream 1; table) is reflected by an increase in Al concentrations of about 200 $\mu\text{Eq l}^{-1}$, corresponding to the amount of exchanged Na^+ . Watersheds with spruce or other coniferous trees seem to be especially vulnerable to the sea-salt effect because of increased collection of airborne sea-salt particles by the forest canopies. In non-acidified areas, Na ions were exchanged with base cations, particularly Ca^{2+} , so episodic acidification (pH reduction) was not registered.

According to the county environmental authorities, deaths to fish were recorded in four rivers (Songdal, Sira, Kvina and Lygna) in Vest-Agder county in February. Brown trout, sea

trout and the relatively more acid-tolerant fish species brook trout and eel were found among the dead fish. In River Songdal, all year-classes of brown trout and sea trout were found dead. A four- to fivefold increase in Cl^- and highly toxic conditions for fish (high Al concentrations and extremely acid water; pH 4.3–4.4) were measured in these rivers. Also, deaths of Atlantic salmon and/or brown



Locations of monitored rivers (numbers) and precipitation stations (letters). Isopleths for chloride concentrations in precipitation during 18–24 January 1993 are also given.

trout were reported from seven watersheds in Hordaland county and of Atlantic salmon smolts in five hatcheries in Sogn and Fjordane county (see figure).

Sea-salt episodes may be more important for sensitive organisms than previously recognized and may partly explain the problems of salmon populations in rivers of slightly acidified areas in western Norway. The results reported here clearly indicate that critical episodes can also occur in slightly acidified catchments due to sea-salt deposition. Sea-salt episodes followed by death of fish may be the first signs of increasing acidification in coastal areas.

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4. Wright, R. F. et al. *Nature* **334**, 422–424 (1988).
5. Wiklander, L. *Geoderma* **14**, 93–105 (1975).
6. State Pollution Control Authority. *Monitoring of Long Range Transported Air Pollutants*. Rep. 533/93 (SFT, Oslo, 1993) (in Norwegian).
7. *Nitrogen from Mountains to Fjords*. Newsletter 1/1994. (Research Programme coordinated by Norwegian Institute for Water Research, Oslo, 1994).

CHANGES IN CHEMICAL COMPOSITION OF RIVERS BEFORE AND AFTER SEA-SALT EPISODES

Monitored rivers

	Before episode					After episode				
River	pH	SO ₄ [*]	ΔCl	ΔNa [*]	ΔH ⁺	ΔAl	ΔBC [*]	ΔSO ₄ [*]	ΔNO ₃	ΔAlk
1. Tovdal	5.01	63	59	−24	7	9	−2	−7	0	−
2. Mandal	4.95	43	167	−42	5	15	−6	−6	−1	−
3. Lygna	5.88	46	213	−24	12	20	−18	−7	7	−
4. Dirdal	5.19	20	264	−26	1	11	29	−2	10	−
5. Årdal	5.59	23	200	−32	0	1	35	0	3	−
6. Vikedal	5.60	34	110	−20	2	5	0	−9	0	−
7. Etne	6.34	33	62	−17	−	0	14	2	4	0
8. Ekso	6.05	16	183	−28	−	2	5	−7	0	−10
9. Modal	5.35	14	324	−68	1	8	38	−7	8	−
10. Gaula	5.69	18	122	−19	3	5	4	−4	1	−
11. Nausta	5.39	18	206	−30	3	0	18	−8	1	−

Streams in River Bjerkreim (river 12) catchment

Stream	pH	SO_4^*	ΔCl	ΔNa^*	ΔH^+	ΔAl	ΔBC^*	ΔSO_4^*	ΔNO_3	ΔAlk
1. Svela	4.90	49	1,433	–208	23	197	0	–30	27	–
2. Øygard	4.98	41	300	–42	6	29	1	–7	2	–
3. Longa	5.17	44	429	–49	22	23	10	–5	1	–

Units, mEq l^{-1} ; asterisks, concentrations for apparent sea-salt fractions. The sea-salt fraction is assumed to be proportional to the Cl concentration and relative ratio of each ion to Cl in sea-water. Delta values are based on average values for the last three months (monthly samples) before the storms and the first monthly sample after the storm events (February sample). Data from the Norwegian programme for monitoring long-range transported air pollutants⁶ and from the national research programme Nitrogen from Mountains to Fjords⁷.

Hard truths to swallow

Sally M. Horrocks

Protein and Energy: A Study of Changing Ideas in Nutrition. By Kenneth J. Carpenter. Cambridge University Press: 1994. Pp. 280. £30, \$39.95.

THE production of dietary guidelines is an occupational hazard for nutritional scientists: witness the fuss surrounding the publication earlier this month of the British government report *Nutritional Aspects of Cardiovascular Disease*. It is a source of debate and controversy that, as Kenneth Carpenter shows, has a very long history. He begins by tracing the course of changing ideas about the role of protein in human nutrition from the first half of the seventeenth century, long before the word 'protein' itself was coined, through to the work of more familiar figures such as Leibig, Voit, Atwater and Chittenden. After briefly considering the discovery of the essential amino acids, he moves on to discuss the more recent debates over optimal levels of protein consumption. The principal focus here is on the identification of protein deficiency in the diets of people — especially children — in developing countries, on the efforts to address this problem and on the eventual rejection of the scientific basis of these attempts. He also takes a quick look at changing ideas about adult needs for amino acids. Whereas the first half of the book covers material familiar to historians of nutrition, by venturing into the period after the Second World War, Carpenter breaks new and exciting ground, raising many issues that merit further investigation.

Presenting an account of these controversies is not, however, his sole objective. By drawing out the contrasts between the past and the present he seeks to illuminate the nature of the scientific process itself, and invites the reader to make judgements on the state of science today. On the basis of historical sources, he argues that science in the past was an enterprise mainly done by individuals working in isolation and motivated by a disinterested desire to find scientific 'truths'. These truths were established by decisive experiments and sustained until new experiments or ideas led to their

demise. Errors were the result of imperfect experimental procedures, failure to appreciate the significance of results or gaps in knowledge. Scientific knowledge derived from experiment went unchallenged by sociological or economic considerations.

The picture of the scientific enterprise that emerges from the second half of the book is altogether different. Basing his

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The "protein man", a familiar figure to shoppers on London's Oxford Street for many years. His pamphlet, which claimed that dietary protein is the cause of lust, reached its thirty-fifth edition.

account on events he witnessed first-hand as a practising scientist, Carpenter depicts modern scientists as a group "engaged in the passionate pursuit of research grants and professional success". Every scientist is dwarfed by national and international funding agencies, who become the leading actors in his story. Scientists have to cede their previously uncontested sovereignty over the field to accommodate the views of economists, development experts and sociologists, who are also supported by

these institutions. Scientific fact is no longer the result of individual endeavour or a single, decisive experiment, but of the negotiations of committees whose "democratic approach to truth is a contradiction in terms". Nevertheless, this truth becomes embedded unassailably in textbooks that educate the next generation of practitioners. Scientific truth has lost its innocence and purity, and has become tainted by association with forces outside its control.

This picture of modern science accords surprisingly well with that painted elsewhere by historians and sociologists of science, which has recently been attacked vehemently by some practising scientists as unacceptably relativist. However, historians and sociologists regard the developments that Carpenter deplores not as a recent form of corruption of a once pure enterprise but as a fundamental part of the creation of new scientific facts and ideas. There is no reason to believe that the past was so very different. Indeed, Carpenter acknowledges that the persistence of some ideas in the past may well have been related more to the high professional status of their advocates than to sound experimental underpinnings. Also, it is clear from the account of the career of Wilbur Atwater, a founding father of nutrition science in the United States, that many of the factors that Carpenter identifies as distorting the scientific process were as important in the past as they are now. Atwater relied for his initial funding on the US Commission of Fish and Fisheries, whereas his later projects were supported by the Massachusetts Bureau of Labor Statistics. Yet Carpenter, despite his assessment of modern science, rejects suggestions that these connections played any formative part in Atwater's conclusions.

Connecting science past with science present sheds light on the nature of the scientific process itself, Carpenter argues. His book does indeed achieve this aim, but not necessarily in the way he intended. Rather than showing how the personal, professional and institutional concerns of modern scientists may have diverted them from the objective search for truth that their predecessors pursued, he instead unwittingly invites us to see these as integral parts of the scientific process, as components of scientific truth itself. □

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Not far out

Malcolm Longair

The Farthest Things in the Universe. By Jay M. Pasachoff, Hyron Spinrad, Patrick S. Osmer and Edward Cheng. Cambridge University Press: 1994. Pp. 104. £19.95, \$29.95 (hbk); £11.95, \$19.95 (pbk).

THE dustjacket of this little book claims: "This survey offers an exceptional opportunity for the general audience to share the excitement of today's astronomical research of the early Universe in an accessible and stimulating way". These are encouraging words but one's enthusiasm is slightly dampened on reading the foreword. The book originates from a series of popular lectures delivered at a meeting of the American Association for the Advancement of Science in San Francisco in 1989. Two of the original lecturers, Patrick Osmer and Hyron Spinrad, have rewritten their contributions to bring them up to date on the topics of quasars and large-redshift radio galaxies respectively. Edward Cheng has written a completely new chapter on COBE observations of the cosmic microwave background radiation and Jay Pasachoff, the general editor, has provided an introductory chapter describing the large-scale

structure of the Universe and how it is observed. Inevitably, there is a feeling of thinness about the material covered: one suspects that the original lectures provided a much more complete *tour d'horizon* of what is undoubtedly one of the most important areas of modern astrophysical cosmology.

The four chapters contain a lot of excellent material and the general reader can be reassured that, so far as they go, they are authoritative and the views expressed are sound. But there is a big difference between an exciting and stimulating lecture and the hard daylight of the printed word. There is no attempt to provide a systematic, coherent account of what is now known about the early Universe. The reader will look in vain for any discussion of primordial nucleosynthesis and the origin of the light elements, the problems of the origin of the formation of structure in the Universe, what the inflationary model of the early Universe is and why it is taken seriously, the physics of dark matter and why it is believed to be central to the cosmological problem, the contributions of radio, infrared, ultraviolet, X-ray and gamma-ray astronomy to studies of the Universe at large redshifts, and so on. Surely these topics are so relevant to current cosmological research that chapters on them should have been included.

On the positive side, the chapters make clear the central contribution of the new high technologies to recent astronomical achievements. The level of presentation of the material varies considerably throughout. Pasachoff and Cheng make every effort to communicate the essence of their topics in an accessible way but there remains the difficulty of describing physical processes in the very early Universe in a meaningful way to a lay audience. Osmer's article is written for the enthusiast who has already done some homework on quasars and Spinrad's article is more or less aimed at the professional. The general reader will find the going tougher and tougher as the story unfolds, and there is not the crutch of a glossary at the end to support the aspiring enthusiast.

It is crucial that experts in all areas of science reach out to the general public and describe in their own words the full significance of their research. Unfortunately, this book misses a real opportunity to do this. Those who want authoritative reviews of the specific areas discussed will not be disappointed. But those wanting a broader perspective of the key issues of modern astrophysical cosmology will have to look elsewhere. □

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THIS famous photograph by David Malin of Orion's Horsehead Nebula, a dense dark cloud where dust is very abundant and which completely absorbs the light from regions behind it, adorns the cover of the third edition of *The Cambridge Atlas of Astronomy* edited by J. Audouze, G. Israel and J.-C. Falque (Cambridge University Press, pp. 471, \$50, \$75). Well-organized, richly illustrated and superbly presented, this large-format guide to planetary science, astronomy and astrophysics has been extensively revised and updated. It now includes material on the Magellan mission, new sections on Neptune, Pluto and solar-system debris, and a 24-page glossary and index.

Theory into practice

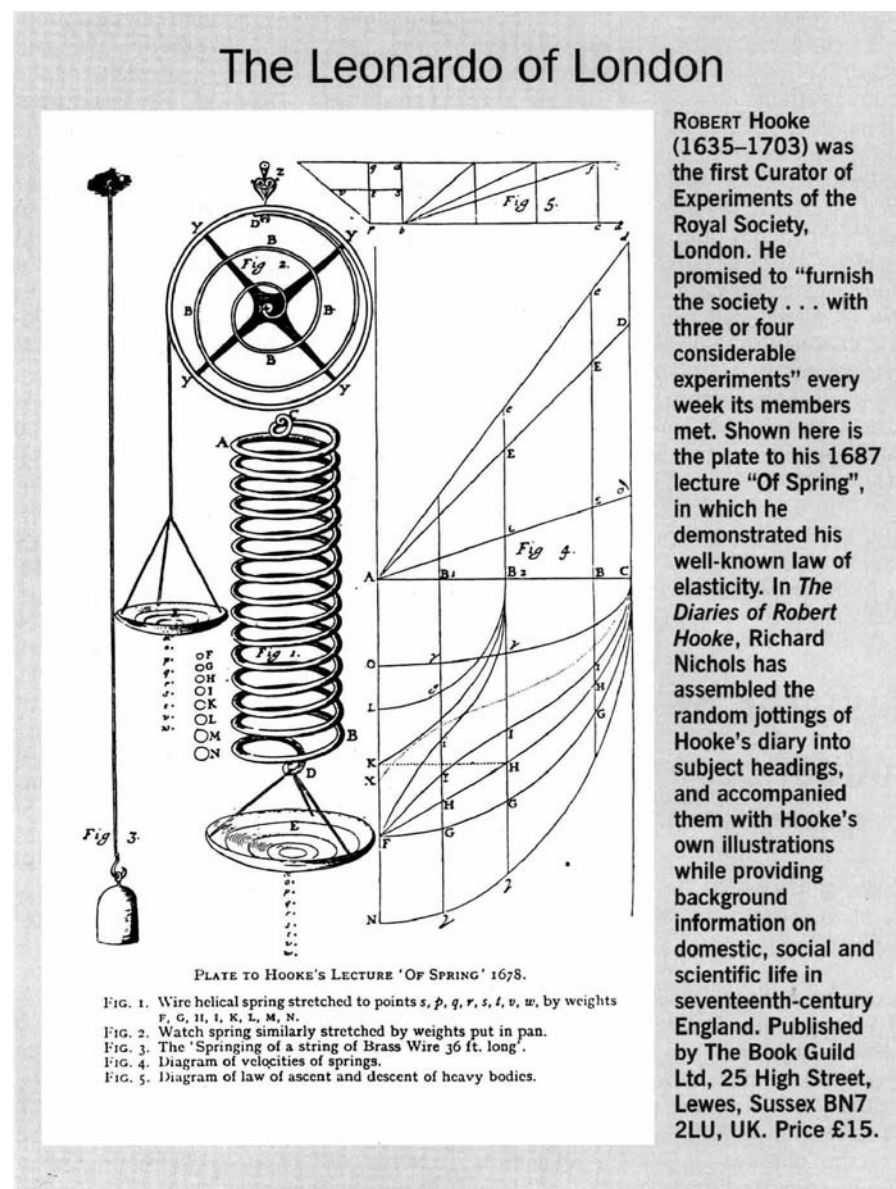
Joel D. Howell

Genetics and Medicine in the United States, 1800–1922. By Alan R. Rushton. Johns Hopkins University Press: 1994. Pp. 209. \$45, £37.

FOR centuries people have noted that offspring tend to favour their parents, an observation that earlier generations of scholars often summarized in the convenient expression 'like begets like'. Today, as every reader of *Nature* must surely know, this older simpler observation has been transformed into the more complex language of molecular genetics, an apparently omnipresent discipline whose persistent promise of widespread clinical use seems to be moving rapidly towards fruition. How should one seek to understand this transition of genetics from general observation to practical tool? In this slim volume, which traces the way in which scientific ideas about heredity and disease made their way into medical discourse in the United States, Alan Rushton shows that apparently new ideas about the clinical relevance of hereditary theories often have much older roots.

He asks an important question: what use could a physician make of the finding that a disease is passed from generation to generation? After examining early nineteenth-century observations of patterns of human inheritance of, for example, haemophilia, he goes on to describe the rediscovery of Mendel's laws of heredity at the beginning of the twentieth century and the ensuing controversy about whether Mendelian theory, which appeared to work well for predicting the inheritance of simple traits in plants and animals, could help to explain the inheritance of diseases in seemingly more complex humans.

Rushton is at his best when exploring the tension between views of disease as having either internal or external causes. Physicians in the early twentieth century debated whether heredity should be thought of as the fundamental cause of disease or merely the source of a tendency to acquire disease, a sort of 'diathesis' that had no effect without the presence of some external stimulus. The microbiological revolution of the late nineteenth century unleashed what at the time seemed to be an endless stream of discoveries linking specific microorganisms to specific human diseases. Microorganisms began to attract more and more attention as a potential external cause of a wide range of ailments. Rushton argues that the microbiological revolution was partly responsible for a shift around the turn of the twentieth cen-



tury away from seeing the causes of disease as primarily hereditary and towards seeing them as primarily environmental. But the shift reflected more than new laboratory science; it also reflected the clinical implications of disease theories. The germ theory of disease, unlike heredity, meant that a clinician had a chance of being able to prevent or treat a patient's disease. But if the cause of disease were exclusively hereditary, then physicians would be able to do far less for their patients. In this sense, the germ theory was far more attractive for clinicians.

The theory of heredity, of course, appeared to hold out the promise of improving the overall health of a human population through selective breeding. Rushton describes US physicians' interest in eugenic science, particularly as promoted by the Eugenics Record Office at Cold Spring Harbor in New York state. Physicians became less enthusiastic about eugenics by the 1920s, he claims, because

of the demonstrated inadequacy of genetics as an explanation of human disease.

Rushton's approach is unabashedly that not of a historian but of "a medical scientist", and he states forthrightly that his work "is not primarily a political or social history". The book is largely based on published medical texts. By relying on this narrow range of sources Rushton ends up listening to the voices of a rather limited community of actors, and as a result some of his conclusions may surprise those acquainted with other literature in the field. Rushton notes for example that the published literature on the use of eugenics to control human reproduction abated after the 1920s, yet an important group of physicians in the United States continued to preach, and to practise, eugenic sterilization for some time. His limited sources also makes it difficult for him to answer his main question about the clinical relevance of genetics: published medical literature, past and present, bears only a modest and variable relationship to the

practice of medicine.

Rushton concludes by connecting the trends he lays out for past generations to today's genetic theories of disease and attempts to predict what the future may bring. Like most scientists and clinicians, he sees genetics as having a growing influence on clinical medicine. But our knowledge of genetics does not automatically, let alone easily, give rise to clinically relevant insights. Rushton correctly identifies the tension between clinicians and scientists as one that will be as important in the future as it has been in the past. His book will help to focus the attention of historians, scientists and clinicians on the important area where theory and practice meet. □

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Spheres of influence

Daniel Kleppner

Rydberg Atoms. By Thomas F. Gallagher. Cambridge University Press: 1994. Pp. 495. £65, £100.

AN atom in which one electron is in a high-lying state, having almost but not quite enough energy to escape, is known as a Rydberg atom. These atoms possess bizarre and intriguing properties. Their diameter can be thousands of times larger than that of normal atoms, their radiation rate thousands of times smaller and their capacity for polarization millions of times greater; they are so fragile that radiation at room temperature can ionize them.

Rydberg atoms have been known since Joseph Balmer guessed the formula for the spectrum of hydrogen in 1883, but they were of only minor interest until the mid-1960s when radio astronomers discovered microwave emission — radio recombination lines — from Rydberg states of hydrogen. What precipitated today's interest, however, was the invention in the next decade of the tunable dye laser and the subsequent introduction of techniques for creating and studying Rydberg atoms in the laboratory. Rydberg atoms rapidly evolved from a novelty into the centrepiece of a large and varied field of atomic research. Although this field is now highly developed, it lacks a definitive overview. Thomas F. Gallagher, a pioneer and key participant in research on Rydberg atoms, has done much to remedy this problem.

He has produced a handbook on the theory and practice of Rydberg atoms,

describing their important physical properties, the relevant experimental and theoretical techniques and many of the mainstreams of research. The basic properties of atoms in Rydberg states and the techniques for producing and detecting them are lucidly presented. Particular attention is paid to their behaviour in electric fields and the process of field ionization. This process is interesting not only because of the physics involved but also because most experiments on Rydberg atoms use field ionization to detect the atoms. Selective field ionization, the detection technique of preference, is described in the general context of the structure of Rydberg atoms in strong electric fields, and adiabatic and non-adiabatic transitions in time-varying fields.

Other important categories include radiative interactions, microwave ionization, structure in magnetic fields, microwave and optical spectroscopy, quantum defect theory, autoionization, double Rydberg states, and collisions. About a third of the book is devoted to collisions, an area in which the author has made seminal contributions. There are chapters on collisions with neutral atoms and molecules, collisions with charged particles, spectral line shifts and broadening, resonant collisions and radiative collisions. The technical presentation is embedded in a lucid and fascinating commentary on the underlying physics.

Altogether, the book provides a reasonably comprehensive overview of the field. However, the important topic of interactions of atoms with the vacuum — cavity quantum electrodynamics and the Casimir force — is not discussed, although black-body radiation effects are covered. Also omitted is the study of semiclassical behaviour in regular and irregular systems — quantum chaos — for which Rydberg atoms have provided model test systems.

Rydberg Atoms is handsomely produced with many illustrations. The index is adequate though not exhaustive. The references to the literature are numerous, including many review articles on particular aspects of research. The book will undoubtedly be the standard reference work in the field for many years to come. As such, it should be invaluable both to experienced researchers and to graduate students. Furthermore, it constitutes a valuable resource for teachers of quantum mechanics and atomic physics, as Rydberg atoms are ideally suited to illustrating the role of scaling laws and a wide variety of basic phenomena such as adiabatic and non-adiabatic behaviour, tunnelling and radiative interactions. □

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New textbooks

Physical Chemistry by P. W. Atkins (3rd edn). Oxford University Press, £50 (hbk), £24 (pbk).

Inorganic Chemistry by D. F. Shriver, P. W. Atkins and C. H. Langford. Oxford University Press, £48 (hbk), £19.50 (pbk).

Biological Chemistry: Inorganic Elements in the Chemistry of Life — An Introduction and Guide by W. Kaim and B. Schwederski. Wiley, £19.95 (pbk).

Models in Biology: Mathematics, Statistics and Computing by D. Brown and P. Rothery. Wiley, \$39.95 (pbk).

Air Pollution and Climate Change: The Biological Impact by A. R. Wellburn (2nd edn). Longman, £17.99 (pbk).

The Key to Earth History: An Introduction to Stratigraphy by Peter Doyle, Matthew R. Bennett and Alistair N. Baxter. Wiley, £12.95 (pbk).

Principles of Conservation Biology by Gary K. Meffe and C. Ronald Carroll. Sinauer/W. H. Freeman, £29.95 (hbk).

An Introduction to Global Environmental Issues by Kevin T. Pickering and Lewis A. Owen. Routledge, £50 (hbk), £15.99 (pbk).

Environmental Science for Environmental Management edited by Timothy O'Riordan. Longman, £17.99 (pbk).

Vertebrate Zoology: An Experimental Field Approach by Nelson G. Hairston Jr. Cambridge University Press, £17.95, \$34.95 (hbk).

Analysis of Vertebrate Structure by Milton Hildebrand (4th edn). Wiley, \$46 (hbk).

Bacterial Systematics by Niall A. Logan. Blackwell Scientific, £18.95 (pbk).

Prokaryotic Genetics: Genome Organization, Transfer and Plasticity by Francoise Joset and Janine Guespin-Michel. Blackwell Scientific, £24.95 (pbk).

Genetics of Bacterial Virulence by Charles J. Dorman. Blackwell Scientific, £29.95 (pbk).

Principles of Gene Manipulation: An Introduction to Genetic Engineering by R. W. Old and S. B. Primrose (5th edn). Blackwell Scientific, £19.50 (pbk).

Introduction to Modern Virology by N. J. Dimmock and S. B. Primrose (4th edn). Blackwell Science, £19.50 (pbk).

Medical Virology by David O. White and Frank J. Fenner (5th edn). Academic, \$95 (hbk).

Developmental Biology by Scott F. Gilbert (4th edn). Sinauer/W. H. Freeman, £29.95 (hbk).

Gene inhibition using antisense oligodeoxynucleotides

Richard W. Wagner

Antisense oligodeoxynucleotides (ODNs) have great promise as agents for the specific manipulation of gene expression. Until recently, nonspecific effects of ODNs often confounded the interpretation of antisense studies. Improvements in ODN chemistry and cellular delivery techniques now allow for more potent and specific gene inhibition. This review critically evaluates recent progress in the development of antisense ODNs.

ANTISENSE oligodeoxynucleotides (ODNs) offer the potential to block the expression of specific genes within cells. The development of effective antisense techniques will allow researchers to address questions about gene function and may also lead to new therapies for many human diseases. Inhibition of gene expression by antisense ODNs relies on the ability of an ODN to bind a complementary messenger RNA sequence and prevent translation of the mRNA (reviewed in ref. 1). Antisense effects of ODNs in mammalian cells have been reported in numerous tissue culture experiments (reviewed in refs 1–3), and in several recent *in vivo* studies^{4–26}. Clinical trials are now in progress to evaluate the therapeutic potential of antisense ODNs in several human diseases, including acute myelogenous leukaemia⁶ and infection by human immunodeficiency virus-1²⁷, cytomegalovirus (CMV)²⁸ and human papillomavirus²⁷.

There are, however, many biological effects of ODNs that cannot be attributed to antisense mechanisms alone (reviewed in refs 1, 2). Such effects may limit the conclusions that can be drawn from antisense experiments, and may also compromise the usefulness of antisense ODNs as therapeutic agents. This review discusses the degree to which antisense ODNs can cause specific inhibition of target genes, and summarizes recent advances that have led to the development of more potent and specific antisense methods.

Nonspecific effects of ODNs

Despite numerous reports of apparent antisense inhibition of gene expression in cultured cells, only in a few cases has specific inhibition been rigorously demonstrated. In many studies, specificity has been inferred from the biological effects of antisense

as compared to control ODNs, without measuring levels of target RNA or proteins to evaluate specificity. Without such controls, however, it is difficult to exclude the possibility that an observed effect may be due to actions other than blockage of the intended target RNA. Unintended side-effects could potentially occur through a number of mechanisms. Antisense ODNs have been shown in *Xenopus* to cause cleavage of imperfectly matched target sites²⁹. Sequence-specific and nonspecific binding of ODNs to small molecules³⁰ and proteins^{31–33} have been described. Nonspecific cellular activation of the SP1 transcription factor by phosphorothioate ODNs was recently reported³⁴. ODNs can also inhibit viral infection by non-antisense mechanisms that are not fully understood, but which may include interference with absorption, penetration or uncoating^{33,35,36}. Finally, nucleosides and nucleotides, the degradation products of ODNs, can affect cell proliferation and differentiation^{37,38}. Because ODNs are degraded both intra- and extracellularly^{39–41}, these breakdown products may account for some of their observed effects, especially at the high concentrations used in most tissue culture experiments.

Caution is therefore required when interpreting antisense studies that do not contain a series of controls to demonstrate specific gene inhibition. The importance of well controlled assays is underlined by reports of difficulties^{42–44} in reproducing effects reported in previous studies. In some cases, the biological effect was reproduced, but inclusion of controls ruled out an antisense mechanism^{42,44}; in others, the effects could not be repeated⁴³, possibly because of differences in cell lines, culture conditions or ODN purity. The concerns raised above suggest that at least some published reports of antisense effects may be incorrect.

There is thus a need for the development of new antisense methods that are more potent, reliable and specific than those used in previous studies.

Controls for measuring antisense effects

In general, the most convincing demonstrations of antisense gene inhibition are those in which antisense mechanisms are meticulously isolated from the nonspecific biological effects of ODNs. Ideal antisense experiments include the following elements.

- Direct measurement of the target RNA or protein levels as compared to an internal control RNA or protein: RNase H hydrolyses the RNA strand of a RNA-DNA duplex and is likely to be responsible for the antisense effects of 2'-deoxy-ODNs. Thus, degradation of the target RNA may be a good assay for gene inhibition. Direct measurements of effects on protein levels may be more problematical, because if protein turnover is slow, some time is needed before a reduced rate of synthesis is detectable as a decrease in the total amount of protein (this applies to both target and control proteins). Another problem arises in cases where the target

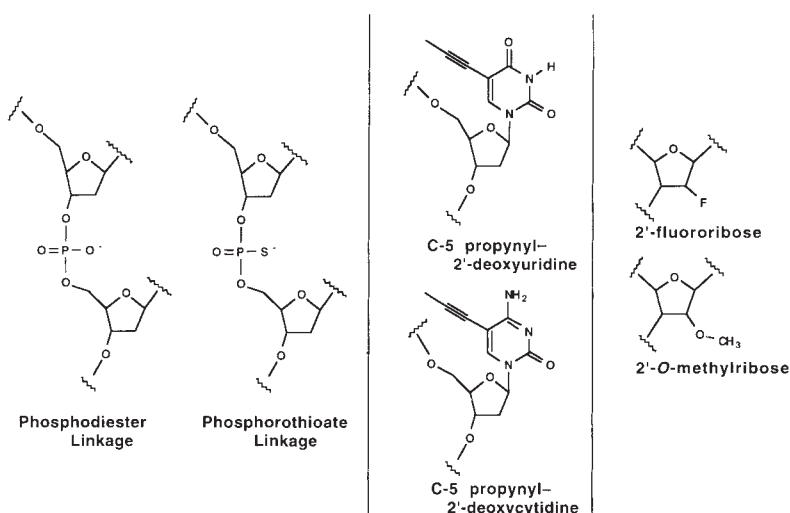


FIG. 1 Structures and modifications of ODN internucleotide linkages, bases and sugars.

protein regulates synthesis of other proteins (as for instance with cell-cycle proteins); in such cases, selection of appropriate controls is difficult, and the best control may be to use several active antisense ODNs targeted to the same RNA as a demonstration of specificity. These common situations, along with inappropriate choice of controls, can make the evaluation of reported antisense effects very difficult.

- **Careful choice of target sequence:** Many investigators have chosen to target the translation initiation site of a mRNA on the assumption that this region is important and accessible. Recent studies indicate that most regions of the mRNA are in fact accessible to ODNs^{42,45,46}, except for those with strong secondary structure⁴⁷. The relative efficacy of different sites, however, appears to depend on the chemistry of the ODN modification; when 15 2'-deoxyphosphorothioate ODNs were tested, only one sequence showed relatively potent effects, whereas when a C-5 propynyl pyrimidine modification was included, the majority of sequences show potent antisense effects^{42,47}. The reasons for these effects are not well understood, and the optimal target sites must therefore be selected empirically. To avoid biasing the outcome of the experiment by the choice of target sequence selection, it is important to show that the same effect is produced by more than one antisense sequence.

- **Mismatched control ODNs:** The choice of control sequences is a critical element in the design of any antisense experiment. Many studies have used a sense ODN complementary to the antisense sequence, whereas others have used ODNs of the same length as the antisense ODN, but composed of a random mixture of all four nucleotides. Because ODNs may be degraded in culture, however, the best control sequences are those that contain the same base composition as the antisense sequence; ideally, the control should differ from the antisense sequence no more than is necessary to prevent specific hybridization.

- **Use of nuclease-resistant ODNs:** ODNs containing phosphodiester linkages are rapidly degraded in most cells, with a half-life typically around 20 min⁴⁰. Thus, unless intracellular concentration can be maintained by continuous delivery, antisense effects may be difficult to observe with phosphodiester ODNs, especially in cases where the target mRNA is being transcribed rapidly. ODNs can be made resistant to nucleases by introducing modifications such as phosphorothioate linkages or 2'-O-allyl groups (refs 1, 40). The importance of stability is confirmed by a study that compared phosphorothioate ODNs with phosphodiester ODNs of identical sequence; in this case, the stabilizing modification was essential for high activity⁴².

- **A demonstration of cell permeability:** A major problem with antisense ODNs is their inability to cross cellular membranes efficiently. ODNs can accumulate in the nucleus if microinjected directly into the cytoplasm, but when added to the culture medium, they accumulate in cytoplasmic granules (presumably endosomes and lysosomes) but not in the nucleus (Fig. 2). Although the apparent antisense effects of ODNs have often been cited as evidence that they do in fact escape from these granular compartments, the studies that show the most potent antisense effects have used cell permeabilization techniques to introduce ODNs directly into the cytoplasm. In some of these studies, permeabilization techniques were found to be absolutely necessary (see below).

Specific gene inhibition using antisense ODNs

Several studies have shown that the potency of antisense ODNs can be greatly enhanced by the use of cationic liposomes to deliver the ODNs into cells. For example 100 nM phosphorothioate ODN was sufficient to reduce ICAM-1 protein levels in endothelial cells by 90% when cationic liposomes were used, whereas no effect was seen in the absence of liposomes⁴⁸. Similarly⁴⁹, in the presence of cationic liposomes, a phosphorothioate ODN concentration of 200 nM was sufficient to produce a 50–80% decrease in RNA and protein levels for the target sequence (human procollagen), whereas no effect was seen in

the absence of the liposome, even at a concentration of 25 μ M ODN. The specificity of the antisense effect in this study was confirmed by showing that mouse procollagen RNA and protein (which coexpressed in the mouse NIH3T3 cells and was thus an internal control) was unaffected.

Many studies have explored the use of chemically modified ODNs to improve stability and affinity for RNA (reviewed in ref. 1). Microinjection and cationic liposomes were used to introduce C-5 propynylpyrimidine-2'-deoxyphosphorothioate-modified ODNs (Fig. 1) into various cell types⁴². The C-5 propynyl pyrimidine-modified ODNs had greatly enhanced affinity for RNA, imparted by the C-5 propynyl moiety, and showed potent and sequence-specific gene inhibition. Phosphorothioates without the C-5 propyne group were much less active. Neither type of ODN showed an effect when added to culture medium without permeation enhancers, but in the presence of cationic liposomes, the C-5 propyne-modified ODN caused a complete loss of the target protein (SV40 large T antigen) at a concentration of 5 nM. The C-5 propyne group was also effective when ODNs were targeted to the *rev* response element of the HIV *env* transcript (where they presumably block binding of *rev* protein

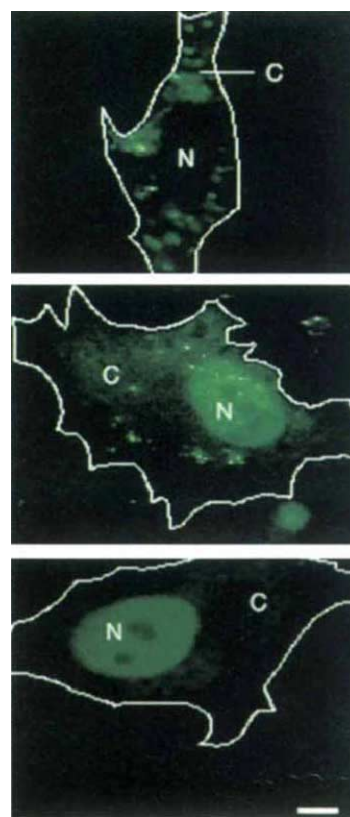


FIG. 2 Uptake of fluorescein-labelled phosphorothioate ODNs into African green monkey kidney cells by endocytosis, cationic liposomes and microinjection. Top, ODNs were incubated with cells in Dulbecco's modified Eagles medium containing 10% fetal bovine serum at 50 μ M for 4 h. Cells were washed and viewed live using fluorescence confocal microscopy as described⁴². All of the cells showed cytoplasmic localization of ODNs in granular compartments. Middle, ODNs were complexed with cationic liposomes at a final media concentration of 250 nM ODN and 10 μ M cationic liposome as described⁴². The complex was incubated with cells for 4 h, and the cells were washed and viewed live. 90% of the cells showed nuclear localization of fluorescent ODN. Bottom, ODNs were microinjected into the cytoplasm of cells at a needle concentration of 25 μ M as described⁴². The cells were incubated at 37 °C for 4 h and the cells were viewed live. All of the injected cells showed nuclear localization of the ODN. Phosphorothioate ODNs were labelled on the 5'-end with fluorescein and purified as described⁴⁰. Confocal microscopy was as described⁴⁰. C, Cytoplasm; N, nucleus; white outline, cell boundary; scale bar, 10 μ m.

to its target site)⁴⁷. Modifications that increase the affinity for RNA, namely 2'-O-methyl or 2'-fluoro groups (Fig. 1), enhance the ability of antisense ODNs to block *c-ras* protein expression, provided that at least 5 consecutive 2'-deoxyribose residues are present to act as a substrate for RNase H.

Thus, chemically modified ODNs are potentially effective antisense agents. Their resistance to nucleases and enhanced binding affinities mean that they can be used at low doses, with correspondingly reduced side-effects. Many of these chemically modified ODNs, however, are inefficient at entering cells, and so special delivery methods are essential if their potential is to be realized.

Therapeutic potential of antisense ODNs

Although several groups have reported apparent antisense effects in whole animal models, many questions regarding the specificity of the observed biological effects remain unanswered. Recent studies have reported inhibition of restenosis in rats^{4,7,12,13,17,18} and pigs²⁶, and inhibition of leukaemic cell proliferation²⁵ and tumour growth^{9,10,16,23,24} in mice. Although each of these studies demonstrated biological effects, in no case did they include quantitative data on reduction of protein and RNA in the targeted cells. A few examples are discussed below.

A phosphorothioate ODN targeted to *c-myb* mRNA was reported to inhibit restenosis in a rat model of balloon angioplasty¹⁷. Although a decrease in *c-myb* RNA was observed, this was measured 2 weeks after the treatment, and may therefore have been an indirect consequence of reduced cell proliferation. It has recently been suggested that inhibition of smooth muscle proliferation *in vitro* is generally nonspecific^{1,51}, and so it will be important to establish the specificity of potential antisense effects *in vivo*.

Growth of *tax*-transformed tumour cells is inhibited *in vivo* by ODNs targeted to NF- κ B or *tax* RNA^{9,10}. Although the level of target protein was reduced *in vivo*, no decrease in target RNA levels was detected in cultured tumour cells. Because the effects of 2'-deoxy ODNs are normally dependent on RNase H-mediated degradation of the target RNA^{42,50,52}, and because phosphodiester-containing ODNs are rapidly degraded inside cells⁴⁰, rigorous controls for sequence specificity are required before it can be concluded that tumour inhibition observed in these

studies is due to antisense effects rather than to nonspecific effects of the type discussed above.

Three studies have tested the use of cell permeabilization methods *in vivo*. In one study¹⁶, a phosphorothioate ODN targeted to the p120 protein was shown to inhibit the growth of human amelanotic melanoma ascites tumours in mice. The effect depended on the use of cationic liposomes, although neither RNA nor protein levels were examined directly. Two other studies reported inhibition of restenosis in rat arteries by phosphorothioate ODNs targeted to PCNA, *cdc2* kinase or *cdk2* kinase^{12,13}. ODNs were delivered by complexing with liposomes containing a viral coat protein. In the absence of liposomes, restenosis was not inhibited. A decrease in target RNA within the treated arteries was reported, although the reverse-transcribed polymerase chain reaction (PCR) assays did not include internal controls to make them quantitative (for examples of quantitative PCR assays see refs 49, 53).

Thus, despite the encouraging biological effects seen in the above studies, in no case has an antisense mechanism been clearly demonstrated with the rigour that has been applied to recent culture studies. There is an urgent need for further studies, with stringent controls for non-antisense effects, in order to make a critical evaluation of the *in vivo* activity of antisense ODNs.

The emerging antisense picture

Antisense ODNs show great promise as agents for blocking specific gene expression. Chemical modifications can enhance both their stability and their potency. ODNs are, however, unable to enter most cell types with high efficiency, and so some form of delivery system is essential. With the use of careful controls and cell permeabilization techniques, more potent antisense ODNs have been developed, and the ability of antisense ODNs to produce sequence-specific gene inhibition is now well established. These advances have paved the way for a critical evaluation of the effectiveness of antisense ODNs *in vivo*, and such studies should eventually lead to the development of improved methods in antisense therapy for human diseases. □

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Crystal structure at 2.2 Å resolution of the MHC-related neonatal Fc receptor

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The three-dimensional structure of the rat neonatal Fc receptor (FcRn) is similar to the structure of molecules of the major histocompatibility complex (MHC). The counterpart of the MHC peptide-binding site is closed in FcRn, making the FcRn groove incapable of binding peptides. A dimer of FcRn heterodimers seen in the crystals may represent a receptor dimer that forms when the Fc portion of a single immunoglobulin binds. An alternative use of the MHC fold for immune recognition is indicated by the FcRn and FcRn/Fc co-crystal structures.

RECEPTORS for the Fc portion of immunoglobulins play a number of important roles in the immune system, including effector functions such as antibody-dependent cytotoxicity and phagocytosis, and transport of immunoglobulin across layers of tightly joined cells¹. One class of Fc receptors (FcRn) mediates transfer of maternal IgG to the newborn, providing humoral immunity to the neonate before the development of a fully functional immune system². Before weaning, suckling mice and rats express a heterodimeric FcRn in the proximal small intestine which binds maternal IgG at the pH of milk in the gut (pH 6.0–6.5). FcRn/IgG complexes are transcytosed across the cell to the basolateral membrane that faces the serosal fluid³, where the maternal IgG dissociates at the pH of blood (pH 7.5). A similar heterodimeric Fc receptor is found in the fetal yolk sac of rats⁴ and a human homologue of FcRn has been discovered in the placenta, where it is presumably involved in transfer of maternal IgG to the fetus⁵.

Molecular cloning of the rodent and human FcRn heavy chains revealed a surprising similarity in the organization of domains and their sequence to the heavy chains of class I MHC molecules^{6–7}. Like the heavy chain of class I molecules, the heavy chain of FcRn consists of three domains, $\alpha 1$, $\alpha 2$ and $\alpha 3$, followed by a transmembrane region and a small cytoplasmic domain. FcRn heavy chains are non-covalently associated with β_2 -microglobulin ($\beta 2m$), the class I light chain⁸. So far, the FcRn family of Fc receptors appear to be unique in their similarity to MHC molecules; other Fc receptors, including Fc γ RI, Fc γ RII, Fc γ RIII, Fc ϵ RI and the poly-Ig receptor, are composed of tandem repeats with sequences similar to immunoglobulin domains¹.

The functional basis for the similarity between FcRn and MHC molecules is not understood. Class I MHC molecules bind short peptides for presentation to T cells⁹ and interact with T-cell antigen receptors and CD8, whereas FcRn binds an immunoglobulin ligand. Crystal structures of MHC class I molecules have revealed that these proteins are folded into a shape ideally suited for peptide binding^{10–15}. Two long α -helices form the sides of a deep groove within the $\alpha 1$ and $\alpha 2$ domains of the heavy chain, and a β -pleated sheet forms the bottom. The groove accommodates octamer and nonamer peptides derived from foreign and self proteins in an extended conformation^{12–15}. $\beta 2m$

and the $\alpha 3$ domain of the heavy chain are underneath the $\alpha 1$ and $\alpha 2$ domains, supporting them in a position to be recognized by a T-cell receptor on another cell. The similarity between FcRn and MHC class I molecules is greatest in the $\alpha 3$ domain (35–37% identity as compared to 27–30% in $\alpha 1$ and 22–29% in $\alpha 2$)⁶.

To investigate how FcRn recognizes Fc, as well as how evolution has exploited the MHC structural motif for different immunological recognition purposes, we crystallized¹⁶ and solved the structures of the extracellular portion of the rat FcRn heterodimer and of an FcRn/Fc complex¹⁷. We find that FcRn is indeed structurally similar to MHC molecules, not only in the conservation of $\alpha 1/\alpha 2$ domain topology of two helices that span a single β -sheet, but also in having the same relative orientation of the heavy and light chains. However, FcRn cannot bind peptides owing to rearrangements of its α -helices, and the counterpart of the MHC peptide-binding groove in FcRn is filled with side chains. To our knowledge, the structures of FcRn alone and of its complex with Fc¹⁷ offer the first view of an Fc receptor structure and its interaction with immunoglobulin, and raise questions concerning the evolutionary relationship between the FcRn family of receptors and MHC molecules.

Structure determination

We previously reported the expression, purification and crystallization in space group $C22_1$ of the soluble extracellular portion of rat FcRn¹⁶, which is composed of residues 1–269 of the FcRn heavy chain associated with rat $\beta 2m$ (99 residues). Soluble FcRn binds IgG and Fc fragments of IgG with the expected physiological pH dependence. The structure determination involved use of two other crystal forms of FcRn in spacegroups $P3_121$ and $P2_1$. The $P3_121$ structure, containing two heterodimers in the asymmetric unit, was initially determined to 3.0 Å by multiple isomorphous replacement (MIR) phasing using two heavy-atom derivatives (Table 2) and two-fold non-crystallographic symmetry averaging¹⁸ (Fig. 1a). The model was refined to 3.0 Å using the program X-PLOR¹⁹.

Because higher-resolution data were available from cryopreserved²⁰ $C22_1$ crystals, the structure was solved again in this space group (Table 2). Molecular replacement phases generated from the $P3_121$ coordinates were used to locate the positions of a heavy-atom derivative in $C22_1$ and the structure was completely rebuilt in symmetry-averaged single isomorphous replacement (SIR) maps which included no information from the $P3_121$ model. Refinement was completed to 2.2 Å (Table 2 and Fig. 1b) using protocols that reduced the statistically independent R_{free} parameter²¹, and the model was verified using simulated annealing omit maps²² generated for six distinct

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TABLE 1 Residues at interdomain interfaces and interface of FcRn dimer of heterodimers

$\alpha 1\alpha 2/\beta 2m$				$\alpha 3/\beta 2m$			
FcRn	H-2K ^b	FcRn	H-2K ^b	FcRn	H-2K ^b	FcRn	H-2K ^b
$\alpha 1\alpha 2$		$\beta 2m$		$\alpha 3$		$\beta 2m$	
His 10	Phe 8	Ile 1	Ile 1	Arg 189	His 192	Gln 8	Gln 8
Ala 12	Thr 10	His 31	His 31	Thr 199	Arg 202	Tyr 10	Tyr 10
Val 14	Val 12	Pro 32	Pro 32	(Ala 201)	Trp 204	Ser 11	Ser 11
Trp 25	Met 23	Pro 33	Pro 33	Phe 203	Leu 206	Arg 12	Arg 12
Thr 27	Val 25	Asp 53	Asp 53	Ser 227	Val 231	His 13	His 13
Trp 29	Tyr 27	Leu 54	Met 54	Ser 228	Glu 232	Pro 14	Pro 14
Gln 34	Glu 32	Ser 55	Ser 55	Gly 229	Arg 234	Pro 15	Pro 15
Thr 37	Arg 35	Phe 56	Phe 56	Pro 230	Pro 235	(Phe 22)	Ile 22
Thr 91	Thr 94	Trp 60	Trp 60	Asn 231	Ala 236	Asn 24	Asn 24
Gln 93	Gln 96			Gly 232	Gly 237	Tyr 26	Tyr 26
Leu 95	Ile 98			Asp 233	Asp 238	(Tyr 28)	Ser 28
Val 111	Gln 115			His 237	Gln 242	(Tyr 63)	Tyr 63
Ala 113	Ala 117			Trp 239	Trp 244	Leu 65	Leu 65
						Asp 98	Asp 98
						Met 99	Met 99

Total buried surface area at the interfaces: $\alpha 3/\beta 2m$: 1,420 Å²
 $\alpha 1\alpha 2/\beta 2m$: 1,585 Å² $\alpha 3/\beta 2m$: 1,420 Å²

$\alpha 1\alpha 2/\alpha 3$				Dimer of FcRn heterodimers	
FcRn	H-2K ^b	FcRn	H-2K ^b	$\alpha 3$	$\beta 2m$
$\alpha 1\alpha 2$		$\alpha 3$			
(Gly 31)	Asp 29	(Glu 180)	Asp 183	Arg 189	Asp 17
Ala 32	Asp 30	Tyr 206	Tyr 209	Pro 190	Glu 74
Gln 33	Gln 31	Pro 207	Pro 210	Gly 191	Glu. 89
Trp 178	Arg 181	Pro 208	Ala 211	Asn 192	Pro 90
Lys 179	Thr 182	Glu 259	Glu 264	Ser 193	Lys 91
				Asn 225	Thr 92
				Leu 241	Thr 94
				Glu 243	Asp 96
				Lys 245	
				Arg 246	
				Asp 248	
				Glu 249	
				His 250	
				His 251	
				Trp 239	
				NAG (attached to Asn 225)	

Total buried surface area at the interfaces: $\alpha 1\alpha 2/\beta 2m$: 707 Å² FcRn/FcRn: 2,136 Å²

Comparison of the residues at the inter-domain interfaces of FcRn and H-2K^b, and list of residues at the interface of the dimer of FcRn heterodimers observed in the three FcRn crystal forms and in the FcRn/Fc co-crystal¹⁷. Residues are listed in numerical order. Residues at the class I interdomain interfaces are those defined previously for the HLA-A2 structure²⁷. In the comparison between FcRn and H-2K^b, residues listed in brackets do not take part in the interaction in one molecule, but form part of the interface in the other. Residues are classified to be at the interface of the dimer of heterodimers if an atom from the residue is within 3.5 Å of a residue on the other molecule or hydrogen-bonds to a bridging water molecule. Buried surface areas between interfaces were determined using X-PLOR¹⁹ with a probe radius of 1.4 Å. NAG, N-acetylglucosamine.

regions covering the heavy chains of the three heterodimers in the asymmetric unit.

A molecular replacement solution of the P2₁ crystal form using the P3₂1 model was verified using calculated phases to locate the positions of heavy atoms in a K₂PtCl₄ derivative (Table 2).

Comparison with MHC molecules

The overall structure of FcRn is similar to that of class I molecules (Fig. 1c). The $\alpha 1$ and $\alpha 2$ region of both FcRn and class I molecules resembles a platform composed of eight antiparallel β -strands forming a single β -sheet topped by two antiparallel α -helices (Fig. 1d). In the central part of the platforms of FcRn and H-2K^b (refs 13, 14), α -carbon atoms of 47 of the 180 residues superimpose with an r.m.s. deviation of 0.62 Å. The superposition is best in the region of the six central strands of the β -sheet and the loops between strands 2 and 3 and strands 6 and 7. The loops connecting strands 1 and 2 and connecting strands 5 and 6 have different conformations from their class I counterparts. Another difference between the class I and FcRn struc-

tures involves strands 7 and 8 of the $\alpha 2$ domain, which are separated from the rest of the β -sheet in FcRn. In class I molecules, the two spanning α -helices are separated by a groove ~25 Å long¹⁰. In all crystal structures of class I molecules solved so far, the peptide-binding groove is occupied by either a mixture of endogenous peptides or by a single defined peptide. Owing to an overall repositioning of the $\alpha 1$ helix and bending of the C-terminal portion of the $\alpha 2$ helix, the FcRn helices are considerably closer together. Near the middle of the FcRn platform, the $\alpha 2$ helix moves over to fill up the space between the helices. Surface representations of the tops of the FcRn and the class I $\alpha 1$ and $\alpha 2$ domains (Fig. 2a) show that there is no continuous groove in FcRn. This correlates with biochemical results showing that soluble FcRn does not contain endogenous peptides²³, and no electron density that is unaccounted for by the FcRn sequence is found between the FcRn $\alpha 1$ and $\alpha 2$ helices.

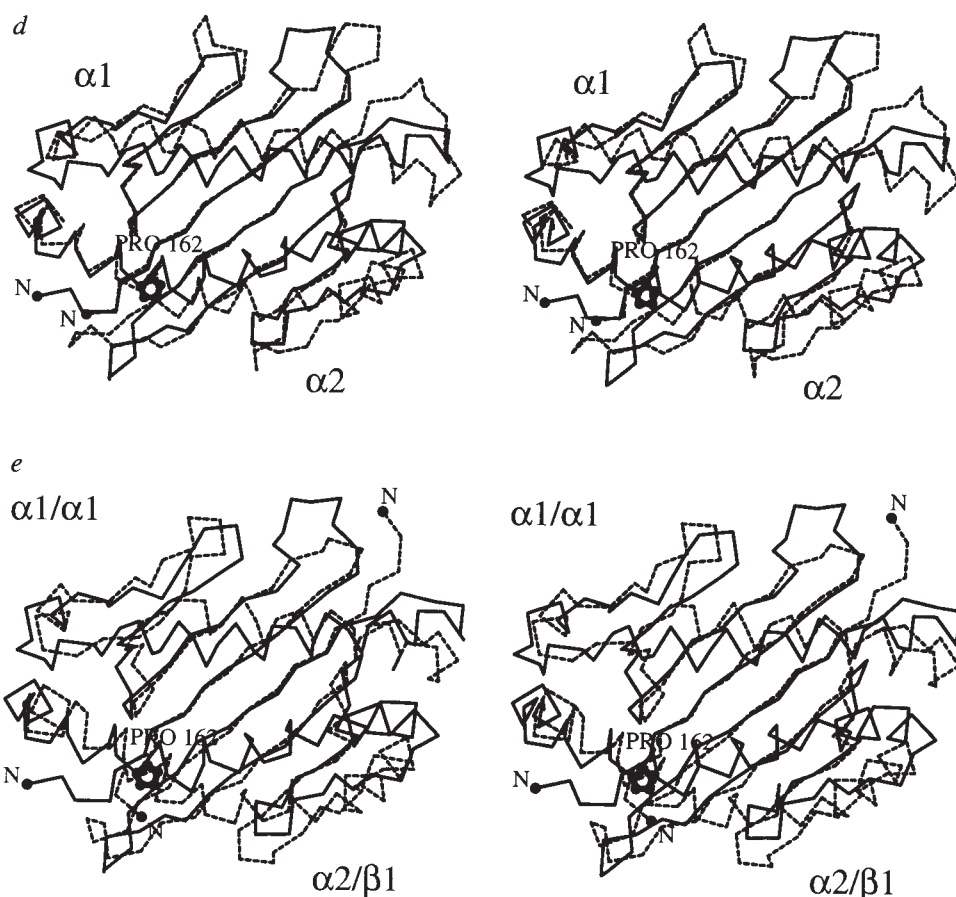
The two FcRn immunoglobulin-like domains, $\alpha 3$ and $\beta 2m$, superimpose closely upon the corresponding domains of class I molecules (0.9 Å r.m.s. deviation for all α -carbon atoms of $\beta 2m$

and 1.4 Å r.m.s. deviation for 81 of 90 α -carbon atoms of the $\alpha 3$ domain; both are compared with the H-2K^b structure^{13,14}). In FcRn, as well as in class I and class II molecules^{10,24}, the two immunoglobulin-like domains interact with non-standard pairing compared with interactions between antibody constant domains. Antibody constant domains are related by a near dyad axis of symmetry²⁵, whereas the FcRn $\alpha 3$ and $\beta 2m$ domains are related by a 156.7° rotation and a 12.5 Å translation. In class I molecules, the $\alpha 3/\beta 2m$ orientation varies between a 146° rotation and a 13 Å translation in HLA-A2 (ref. 10) to a 159° rotation and 13 Å translation in H-2K^b (ref. 13). In class I molecules, $\beta 2m$ stabilizes the heavy-chain structure²⁶ and the large translation along the rotation axis positions it under the $\alpha 1$ - $\alpha 2$ peptide-binding platform, where it makes a number of contacts to the bottom of the $\alpha 1$ - $\alpha 2$ β -sheet²⁷. Many of the heavy chain/ $\beta 2m$ contacts are conserved between FcRn and class I molecules (Table 1). The fourth β -strand of $\beta 2m$ (residues 52–55), which is involved in contacts to the $\alpha 1$ and $\alpha 2$ domains in both FcRn and class I molecules, differs in conformation between the murine and human proteins¹³. The structure of this region of rat $\beta 2m$ more closely resembles the human protein. Compared to class I structures, the FcRn heavy chain makes additional contacts to $\beta 2m$, in that the loop between β -strands 1 and 2 of the $\alpha 1$ domain (residues 16–21) dips downwards to contact $\beta 2m$.

As would be expected from the similar structures of class I and class II MHC molecules, the overall structure of FcRn also resembles a class II MHC molecule^{24,28}. The $\alpha 1$ domain of class II MHC corresponds to the $\alpha 1$ domain in FcRn and the $\beta 1$ domain in class II corresponds to $\alpha 2$ of FcRn (Fig. 1e). In the superposition of FcRn and HLA-DR1 (ref. 28), α -carbon atoms of 59 of 180 residues superimpose with an r.m.s. deviation of 1.4 Å. Unexpectedly, the two outermost strands of the $\alpha 1$ domain of class II molecules are detached from the β -sheet of the platform in a way similar to the two outermost strands of the $\alpha 2$ domain of FcRn, but the affected strands are located at opposite ends of their respective platforms.

Pockets in FcRn and class I grooves

Crystallographic studies of class I molecules revealed that the N and C termini of peptides are accommodated in pockets at each end of the peptide-binding site^{12, 15,27,29}. These pockets (A and F) (Fig. 2c) are mostly lined with residues that are conserved in class I sequences, whereas the intermediate pockets (B to E) contain residues that vary. In pocket A, tyrosines 7, 59, 159 and 171 hydrogen-bond directly or through a water molecule with main-chain atoms of the N-terminal residue of the peptide; in many class I alleles Trp 167 forms part of the opening rim^{12, 15,27,29} (Fig. 2e). The corresponding residues in FcRn are Tyr 9, Tyr 60, Phe 156, His 168 and Arg 164. Although the $\alpha 1$ and $\alpha 2$ helices are probably far enough apart at this side of the FcRn groove to accommodate a peptide amino terminus, and some of the residues forming pocket A in class I molecules are conserved in the FcRn sequence, the side chain of Arg 164 effectively occludes pocket A (Fig. 2b, d). The positive charge of this



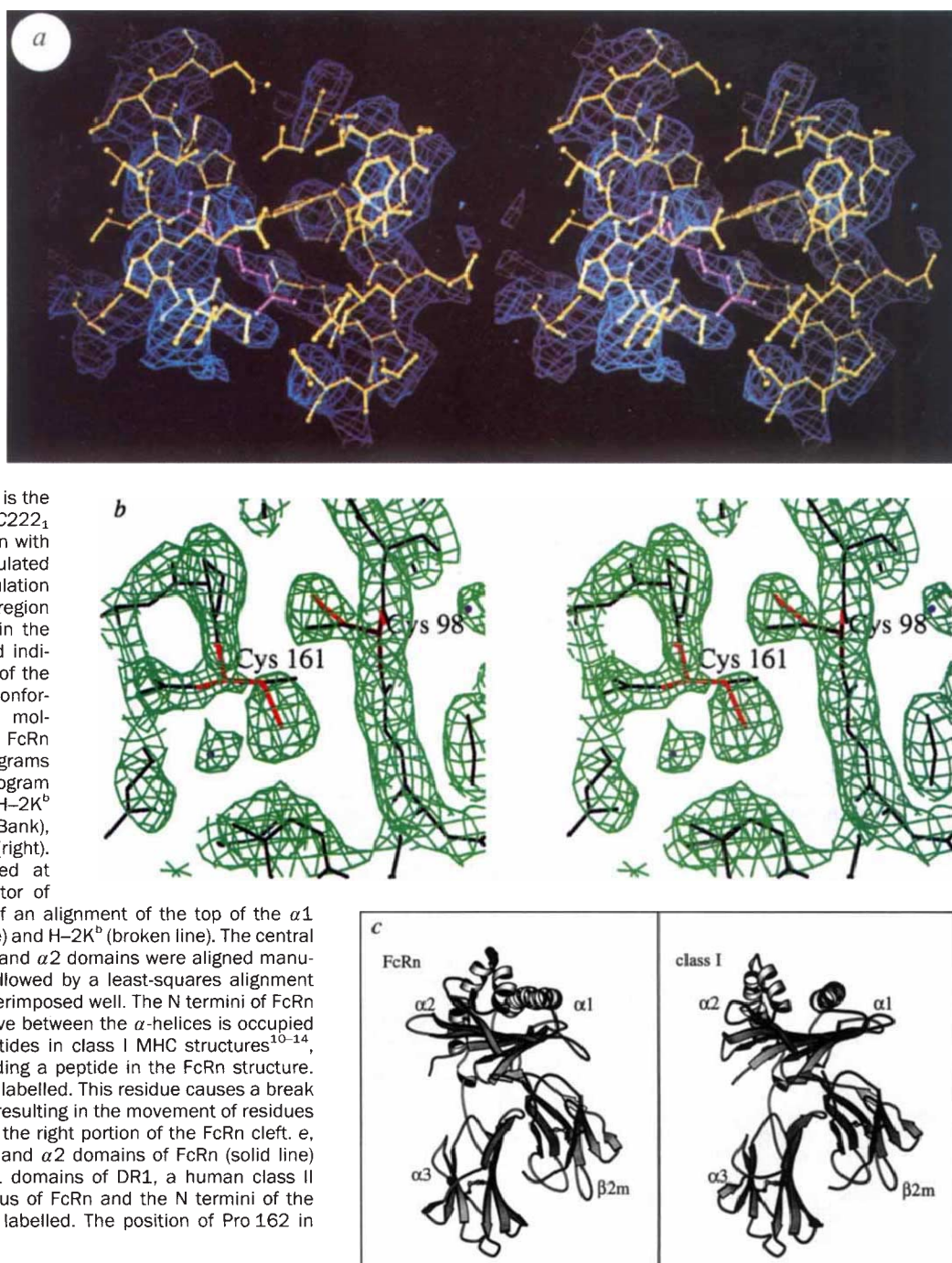
side chain, which is conserved in rat⁶, human⁵ and murine⁷ FcRn sequences, further ensures that the positively charged N terminus of a peptide would be excluded from the site. Remnants of some of the pockets can be seen in the FcRn structure, but of 27 surface-accessible residues constituting the peptide-binding groove of H-2K^b (refs 13, 29), 20 of their counterparts in the FcRn structure are buried (Table 3). At the position of pocket F, the $\alpha 2$ helix comes so close to the $\alpha 1$ helix that only small indentations in the surface structure can be seen (Fig. 2a).

The rearrangement of the FcRn $\alpha 2$ helix compared to class I molecules is due to a break in the helix introduced by the presence of Pro 162 (conserved in rat⁶, murine⁷ and human⁵ FcRn sequences; a valine in class I sequences³⁰). The result is that the 40 amino acids encompassing the N-terminal portion of the $\alpha 2$ helix, the preceding short helix (residues 133–147), and the two outermost strands of the $\alpha 2$ β -sheet (residues 121–132) move almost as a rigid body to close this side of the groove. The binding site for Fc, deduced from the co-crystal structure¹⁷, includes the portion of the FcRn $\alpha 1$ and $\alpha 2$ domains that is moved as a rigid body compared to class I molecules (Fig. 2a). In class I molecules, this part of the structure is involved in few interactions with the rest of the protein, raising the possibility that empty class I molecules could undergo a conformational change similar to their peptide-filled counterparts. However, some differences between the structures of an empty class I molecule and FcRn might be expected, given that FcRn is thermally stable in the absence of bound peptide²³, whereas class I molecules show significantly decreased thermal stability in their empty form³¹.

A dimer of FcRn heterodimers

Although soluble FcRn molecules are monomeric (that is, they consist of one heterodimer) in solution¹⁶, a dimer of FcRn heterodimers is observed in the three crystal forms of FcRn and

FIG. 1 Electron density maps and comparison of the FcRn structure with MHC structures. **a**, 3.0 Å $P3_121$ MIR map after iterative two-fold non-crystallographic symmetry averaging¹⁸. The map is contoured at 1.5σ using a 2.0 Å cover radius around the atoms shown. The superimposed model is the 3.0 Å structure refined using the $P3_121$ data. The map shows the region around Arg 164 (magenta), a residue within the counterpart of the class I pocket A that occludes the pocket in FcRn (Fig. 2d). **b**, 2.2-Å annealed omit map²² contoured at 1.2σ , made using the $C222_1$ structure factors and calculated phases. The superimposed model is the 2.2 Å structure refined using the $C222_1$ data. The part of the model shown with the map was omitted from the simulated annealing refinement before calculation of this map. The map shows the region of the broken disulphide bridge in the $\alpha 2$ domain (Table 2 legend). Red indicates the principal conformation of the cysteine and black, the minor conformation; blue, represents water molecules. **c–e**, Comparison of the FcRn and MHC structures. **c**, Ribbon diagrams (generated with the program MOLSCRIPT⁴⁸) of FcRn (left) and H-2K^b (entry 1VAB in the Protein Data Bank), a murine class I MHC molecule¹³ (right). The H-2K^b structure was refined at 2.5 Å to a crystallographic *R*-factor of 15.4% (ref. 13). **d**, Stereo view of an alignment of the top of the $\alpha 1$ and $\alpha 2$ domains of FcRn (solid line) and H-2K^b (broken line). The central part of the β -sheet within the $\alpha 1$ and $\alpha 2$ domains were aligned manually using interactive graphics, followed by a least-squares alignment of those α -carbon atoms that superimposed well. The N termini of FcRn and H-2K^b are labelled. The groove between the α -helices is occupied with a peptide or mixture of peptides in class I MHC structures^{10–14}, but sterically prevented from binding a peptide in the FcRn structure. The position of Pro 162 in FcRn is labelled. This residue causes a break within the FcRn $\alpha 2$ domain helix, resulting in the movement of residues 121–162 as a rigid body to close the right portion of the FcRn cleft. **e**, Stereo view of the top of the $\alpha 1$ and $\alpha 2$ domains of FcRn (solid line) superimposed on the $\alpha 1$ and $\beta 1$ domains of DR1, a human class II MHC molecule^{24,28}. The N terminus of FcRn and the N termini of the class II $\alpha 1$ and $\beta 1$ domains are labelled. The position of Pro 162 in FcRn is indicated.



in the FcRn/Fc co-crystals¹⁷. Because the binding stoichiometry of FcRn/Fc interactions is two molecules of FcRn bound to a single Fc or IgG (ref. 32), the non-crystallographic dimer may represent a dimer that functions in ligand binding. The dimer (Fig. 3a, b) is formed by interactions between $\alpha 3$ and the symmetry-related $\beta 2m$ and between the two symmetry-related $\alpha 3$ domains (Table 1). A total of 1,068 Å² of buried surface area is contributed from each FcRn molecule, comparable to the 1,242 Å² buried at the interface of the MHC class II dimer²⁴ (our calculation using the refined coordinates from ref. 28). If the observed dimer functions in IgG binding at the cell surface, each FcRn heterodimer must be aligned with its longest dimension roughly parallel to the plane of the membrane (Fig. 3b) rather than perpendicular, which is the orientation believed to be relevant for class I and class II MHC molecules^{10,24}. The dimer is arranged such that the C termini (residue 269) of each

of the truncated FcRn heavy chains are 32 Å apart. There are another seven amino acids before the predicted entrance to the membrane which were removed in the soluble form of FcRn¹⁶. These are followed by a 24-residue, presumably helical, hydrophobic transmembrane region and a 44-residue cytoplasmic domain. If the seven remaining extracellular residues adopt an extended conformation, they span the 23 Å necessary to lift the FcRn dimer off the surface of a membrane (Fig. 3b). This orientation of the receptor, in which it lies roughly flat on the membrane, corresponds to models proposed for the orientation of the IgE receptor FcεRI (ref. 33).

None of the crystal forms of class I MHC molecules shows dimers or higher aggregates of class I heterodimers^{10,15}, but a dimer of class II heterodimers is seen in four crystal forms of HLA-DR1 (refs 24, 28, 34). By contrast to the observed FcRn dimer, the class II dimer is arranged with both molecules orien-

TABLE 2 Data collection statistics

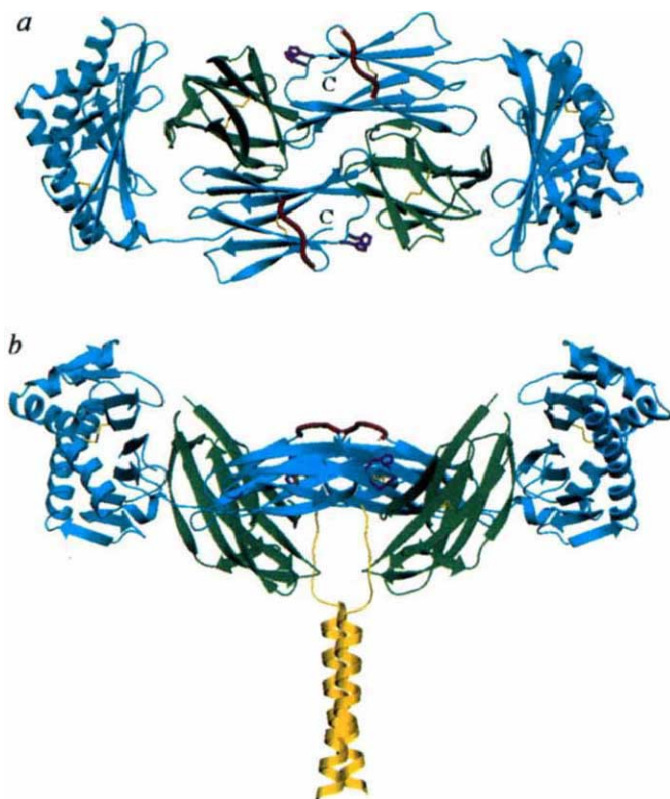
X-ray source/ Temperature/ No. of crystals	Space group/ (mol./AU)*	a (Å) b (Å) (β) c (Å)	Derivative/ Unique†/ Redundancy‡	Resolution (Å)/ R _{merge} (%)/ Complete (%)	Resolution (Å)/ R _{merge} (%)/ Complete (%)	MFID (%)†	R.m.s. f _h /E#	Mean FOM [§]
Rotating anode 22 °C 1	P3 ₁ 21 (2)	114.8 114.8 204.6	Native 28,467 1.8	25–3.0 9.7 76	3.2–3.0 37.5 70			0.27
Rotating anode 22 °C 4	P3 ₁ 21 (2)	114.8 114.8 204.6	HgCl ₂ 18,110 3.0**	25–3.3 14.2 76	3.5–3.3 22.8 34	17.6	0.7	
Rotating anode 22 °C 3	P3 ₁ 21 (2)	114.8 114.8 204.6	EMP 18,153 2.6**	25–3.3 12.5 76	3.5–3.3 23.6 38	17.0	0.7	
CHESS†† –170 °C 2	C222 ₁ (3)	126.5 191.7 149.6	Native 83,821 5.2	25–2.2 7.2 86	2.3–2.2 32.5 49			0.23
CHESS†† –170 °C 1	C222 ₁ (3)	126.5 191.7 149.6	K ₂ PtCl ₄ 35,725 3.9	25–3.0 5.5 98	3.2–3.0 11.8 99	22.2	0.9	
CHESS†† –170 °C 1	P2 ₁ (6)	115.7 164.4 (101.7°) 116.5	Native 71,401 2.4	25–3.0 4.5 83	3.1–3.0 26.6 67			
CHESS†† –170 °C 1	P2 ₁ (6)	115.7 164.4 (101.7°) 116.5	K ₂ PtCl ₄ 40,314 2.6	25–3.5 5.9 74	3.6–3.5 26.5 62	24.2		
Refinement statistics (C222 ₁)								
Resolution		6–2.2 Å						
R _{cryst} ††		21.2%						
R _{free} §§		29.4%						
Number of reflections								
Working set		75,367						
Test		4,060						
Number of scattering atoms		9,612						
R.m.s. deviation from ideality								
Bonds		0.014 Å						
Angles		2.1°						
Average B-factor		49.7 Å ²						
R.m.s. difference of B-factors between bonded atoms		5.0 Å ²						

Crystallization. Expression, purification and crystallization of FcRn in space group C222₁ has been described¹⁶. Trigonal crystals (space group P3₁21; two molecules per asymmetric unit) were grown at 22 °C in hanging drops by combining 2 µl protein solution (36 mg ml^{−1} FcRn, 25 mM PIPES, pH 6.5, 0.02% NaN₃) with 2 µl reservoir solution containing 22% PEG 3350, 5% saturated (NH₄)₂SO₄, 50 mM PIPES, pH 6.5, 0.02% NaN₃. Crystal growth was induced by microseeding. Monoclinic crystals (space group P2₁; six molecules per asymmetric unit) were grown under similar conditions without microseeding and in the presence of 2% saturated (NH₄)₂SO₄.

Heavy-atom derivatives. Crystals were handled and derivatized in 27% PEG 3350, 200 mM NaCl, 50 mM PIPES, pH 6.5 (storage buffer). Phosphate was used in place of PIPES for the preparation of mercury derivatives. P3₁21 HgCl₂ derivative (modified from ref. 40): a solution of 3 mM *N*-acetylhomocysteinethiolactone and 6 mM HgCl₂ in the storage buffer was prepared immediately before soaking of crystals for 24 h. P3₁21 EMP derivative: crystals were reduced in storage buffer containing 20 mM DTT for 12 h, then transferred into storage buffer containing saturated ethylmercuriphosphate (EMP) in the absence of DTT. K₂PtCl₄ derivatives of the C222₁ and P2₁ space groups: crystals were soaked for 12 h in storage buffer containing 2 mM K₂PtCl₄.

Cryocooling. Orthorhombic crystals were transferred over a period of several hours with 3% increments of increasing ethyleneglycol concentration to storage buffer containing 20% ethyleneglycol. The same procedure for cryopreservation of monoclinic crystals was followed using glycerol as a cryoprotectant. Single crystals were mounted using a glass loop⁴¹ and quickly cooled to −170 °C in a nitrogen gas stream using a Siemens LT-2A cryostat. Cryopreserved crystals were screened on a Siemens proportional multiwire area detector mounted on a Siemens rotating anode. Reflections were observed from native crystals to ~3.3 Å resolution (C222₁) and ~3.8 Å resolution (P2₁). Acceptable crystals were transported in a liquid nitrogen dewar to the Cornell High Energy Synchrotron Source (CHESS) for data collection. **Data collection.** Data from native and derivative trigonal crystals were collected at room temperature on an R-Axis IIC X-ray detector mounted on a Rigaku rotating anode (200 µm focal cup; 50 kV, 100 mA). Detector data were integrated using the package provided by the Molecular Structure Corporation. Native and derivative data from cryopreserved orthorhombic and monoclinic crystals were collected at −170 °C at the CHESS F1 beam line (λ = 0.91 Å). Diffraction data were recorded on Fuji HR-III image plates and digitized with a BAS 2000 Fuji scanner. CHESS data were indexed and integrated using DENZO (Z. Otwinowski, personal communication). All data were scaled and further processed using the CCP4 suite⁴². Cryopreserved crystals of both space groups showed an increase in the resolution of usable native data of at least 1 Å at CHESS compared to the usable data from the same frozen crystals using the rotating anode generator. **Phasing, model building and refinement.** P3₁21 crystals: Heavy-atom refinement and phasing was done using the program MLPHARE⁴³. An initial 3.3-Å MIR map in space group P3₁21 (the correct enantiomorph of the space group was determined using anomalous data from the EMP derivative) was calculated using phasing information from the EMP and HgCl₂ derivatives and improved by solvent flattening⁴⁴. Although the initial map was not readily interpretable, a six-dimensional real-space search¹⁰ using the α-carbon coordinates of β2m (entry 2HLA in the Protein Data Bank) yielded a clear solution for the location of one β2m domain. The heavy chain of HLA-A2 fitted well into neighbouring density, and the second FcRn molecule in the asymmetric unit was located using information from the real space search and the positions of heavy atoms in one of the derivatives. Rigid body refinement of the two molecules was followed by real space refinement of the non-crystallographic two-fold axis using the density correlation algorithm in the program RAVE¹⁸. The map was further improved by iterative real-space non-crystallographic symmetry averaging using RAVE¹⁸. The program O (ref. 45) was used for model building and the model refined imposing strict non-crystallographic symmetry to 3.0 Å using X-PLOR¹⁹. C222₁ crystals: the model of the P3₁21 non-crystallographic FcRn dimer of heterodimers was used to identify the location of FcRn in the C222₁ native data using Patterson correlation refinement⁴⁶ and the translation function in X-PLOR¹⁹. One FcRn dimer of heterodimers was located, which produced an *R*-factor after rigid body refinement of 49.4% using all data between 15 Å and 6 Å. Calculated phases using this model allowed identification of the heavy-atom sites of the C222₁ K₂PtCl₄ derivative, which had been previously uninterpretable using difference Patterson techniques. SIR phases improved by two-fold non-crystallographic symmetry averaging¹⁸ yielded an interpretable map at 3.0 Å, but the overall quality was lower than the P3₁21 map at the same resolution. After a six-dimensional real-space search using the α-carbon coordinates of β2m, additional density in the C222₁ map was found to correspond to a third FcRn molecule. The third FcRn molecule is not as well ordered as the two molecules in the dimer of heterodimers (see discussion of B-factors below), and could not be as reliably modelled as the other two heterodimers. The model including three molecules per asymmetric unit was first refined imposing strict non-crystallographic symmetry constraints, followed by multiple cycles of manual rebuilding, simulated annealing and positional refinement using X-PLOR¹⁹. During the final cycles of refinement, non-crystallographic symmetry restraints were released, and the three molecules were refined independently. The correctness of the model was verified by calculation of a real-space *R*-factor on a per-residue basis⁴⁷, the use of an unbiased *R* factor during refinement (*R*_{free}; ref. 21), calculation of simulated annealing omit maps²², and comparison of the main- and side-chain conformations in the model to a database of peptide fragments from well refined structures⁴⁷. Refinement strategies that reduced *R*_{cryst} (the conventional crystallographic *R*-factor) at the expense of *R*_{free} were not pursued. The *R*-factors quoted were calculated without a sigma cutoff. The final model includes 1,189 residues in three FcRn molecules, 614 ordered water molecules, 1 sulphate ion, 5 *N*-acetylglucosamine residues, 2 fucose residues and 3 mercaptoethanol molecules. All residues have allowed θ , Ψ angles in a Ramachandran plot. Seven residues are modelled with alternative conformations: His 251 (on one of the FcRn molecules of the dimer of heterodimers) and the two cysteines within the α2 domain (Cys 98, Cys 161; on all three FcRn molecules). Strong density was observed for sulphur atoms pointing away from the position of the disulphide bridge, and weak density for atoms forming a bridge. The cysteines were modelled with an occupancy of 0.8 for the non-covalent and 0.2 for the covalent conformation. The absence of the disulphide bridge was verified in *F*_o − *F*_c maps and in annealed omit maps²² (Fig. 1b). Electron density corresponding to *N*-linked carbohydrate was observed at Asn 104 and Asn 225 (the P3₁21 electron density maps showed density for carbohydrate at Asn 128 as well) but only a total of 7 monosaccharide units could be reliably modelled, all of which are located on the two FcRn molecules that form the dimer of heterodimers (one

FIG. 3 Dimer of heterodimers found in three crystal forms of FcRn. Ribbon diagrams (generated using SETOR⁵⁰) are shown in two different orientations (differing by $\sim 90^\circ$) for the dimer of heterodimers observed in the FcRn crystals. The FcRn heavy chains are blue, $\beta 2m$ is green, and disulphide bonds are yellow. Heavy-chain residues identified by site-directed mutagenesis that affect the binding affinity of IgG (ref. 35) are highlighted on the dimer structure (His 250 and His 251 (magenta) and the loop containing residues 219–224 (red), the counterpart of the class I CD8 binding loop³⁶). The two FcRn molecules in the dimer are related by a 180.0° rotation in the $P3_121$ crystals, by 176.3° in the $C222_1$ crystals, and by 179.4° , 180.0° and 177.6° in the $P2_1$ crystals (angles relating the six FcRn molecules in the asymmetric unit that form three non-crystallographic dimers). The rotation angles were calculated after refinement of the $P3_121$ and $C222_1$ models (Table 2), or after rigid body refinement of the molecular replacement solution of the $P2_1$ crystals. (All FcRn molecules in the $P3_121$ and the $P2_1$ crystals are related to a partner FcRn by this approximate dyad interaction. In the $C222_1$ crystals, two of the three molecules in the asymmetric unit are related by the dimer interaction, but a third, poorly ordered molecule does not have a dimer related partner.) The same dimer arrangement of FcRn molecules is seen in crystals of FcRn bound to Fc, and its relevance to IgG binding by FcRn is discussed in the accompanying paper¹⁷. Side chains located at the dimer interface are listed in Table 1. Much of the contact is mediated by 16 ordered water molecules within hydrogen-bonding distance ($<3.5 \text{ \AA}$) of both FcRn molecules. *a*, 'Top' view of the dimer with the C termini of each FcRn heavy chain labelled. *b*, Hypothetical model of the FcRn dimer of heterodimers on the surface of the cell. A 'side' view of the dimer is shown, with the 7 amino acids following the C termini of each of the heavy chains of the dimer of soluble FcRn modelled as extended strands (yellow) and the transmembrane regions of both heavy chains modelled as α -helices (yellow). The plane of the membrane is assumed to be perpendicular to the transmembrane helices.



ted with their long axes roughly perpendicular to the membrane, such that the dimerization interface is along the 'side' of the molecule and the two peptide-binding domains are next to each other. It has been suggested that dimerization of class II heterodimers may be a mechanism for initiating signalling pathways in T cells by crosslinking two T-cell receptors²⁴. Similarly, the dimerization of FcRn at the membrane induced by IgG binding could be a signal for endocytosis and subsequent transcytosis of FcRn/IgG complexes¹⁷.

FcRn binding of Fc

Previous work implicated a portion of $\beta 2m$ and residues within the $\alpha 3$ domain in ligand binding³⁵. Within the $\alpha 3$ domain, site-directed mutagenesis showed that mutation of His 250 and His 251 resulted in mutant FcRn molecules with reduced affinities for IgG (ref. 35). The histidines are located at the FcRn dimer interface (Table 1 and Fig. 3*a, b*) so their effect on IgG binding affinity is probably due to participation in FcRn-dimer

formation rather than direct interaction with Fc (ref. 17). Altering the FcRn counterpart of the class I loop which has been identified as the main site of interaction with CD8 (ref. 36) only slightly reduced the affinity of FcRn for IgG (ref. 35). This result suggests that the FcRn/IgG interaction is not similar to class I/CD8 interactions, as might be expected given that Fc and CD8 have very different three-dimensional structures^{37,38}. The visualization of the FcRn/Fc interaction in the co-crystal structure¹⁷ shows that the FcRn counterpart of the class I peptide-binding groove is not involved in ligand binding. Rather, the binding interface on FcRn involves the side of the FcRn molecule (Fig. 2*a*), ruling out analogies between FcRn binding of IgG and MHC/peptide interactions or T-cell receptor recognition of MHC molecules. There is also no resemblance between the binding site on FcRn for Fc and the binding site on a human class II MHC molecule for the superantigen SEB. SEB binds to the $\alpha 1$ domain of the class II molecule³⁴, interacting with the

N-acetylglucosamine at residue 104 in one of the FcRn molecules, two *N*-acetylglucosamines and one fucose on Asn 225 in both FcRn molecules). No carbohydrate is visible at Asn 87 (the other potential *N*-linked glycosylation site) in maps of either space group, but this residue is poorly ordered. The unpaired cysteine at position 48 appears to be blocked by a group that cannot be identified from the electron density. It has been modelled as a mercaptoethanol molecule. The average *B*-factor of all atoms in the three heterodimers is $49.2 \text{ \AA}^2$, corresponding to $42.9 \text{ \AA}^2$ for the two molecules of the FcRn dimer of heterodimers (Fig. 3*a, b*), and $62.4 \text{ \AA}^2$ for the third FcRn molecule. The overall *B*-factor was estimated as $45.4 \text{ \AA}^2$ from a Wilson plot of the native data between 25 $\text{\AA}$ and 2.2 $\text{\AA}$, agreeing with the average *B*-factor of all atoms determined from refinement. The high overall *B*-factor may be the result of the use of high-intensity synchrotron radiation and cryopreserved crystals, which allowed collection of extremely weak high-resolution data. The conformations of the three crystallographically different FcRn molecules in the $C222_1$ crystals are very similar to each other and to the two FcRn molecules in the $P3_121$ crystals (the average r.m.s. deviation for equivalent α -carbon atoms is 0.54 $\text{\AA}$). $P2_1$ crystals: the structure of the $P2_1$ crystals was solved by molecular replacement as described above. The asymmetric unit contains 6 molecules of FcRn, arranged in three pairs of dimers of heterodimers. The solution was verified by using molecular replacement phases to locate heavy-atom sites in the K_2PtCl_4 derivative.

* Mol/AU, number of FcRn heterodimers per asymmetric unit.

† Unique, number of unique reflections.

‡ Redundancy, number of measurements/number of independent reflections.

§ $R_{\text{merge}} (\%) = 100 \times (\sum |I - \langle I \rangle| / \sum I)$, where I is the observed intensity and $\langle I \rangle$ is the average intensity from several measurements.

|| Complete (%) = $100 \times \text{number of independent reflections} / \text{maximum number theoretical}$.

¶ MFID (mean fractional isomorphous difference) = $\sum |F_{\text{ph}} - F_p| / \sum F_p$, where F_p is the protein structure factor amplitude and F_{ph} , the heavy-atom derivative structure factor amplitude.

R.m.s. f_h/E (phasing power), where f_h is the heavy-atom structure factor amplitude and E is the residual lack of closure error.

*† FOM: figure of merit.

** Only reflections with $I/\sigma > 1.0$ were used.

†† CHESS, Cornell High Energy Synchrotron Source.

‡‡ $R_{\text{crystal}} = \sum_a (|F_o - F_c|) / (\sum_a F_o)$, where F_o and F_c are observed and calculated structure factor amplitudes and a represents refinement-set reflections.

§§ $R_{\text{free}} = \sum_t (|F_o - F_c|) / (\sum_t F_o)$, where t represents free-set reflections.

||| Average *B*-factor: average of *B*-factors for all scattering atoms in the structure.

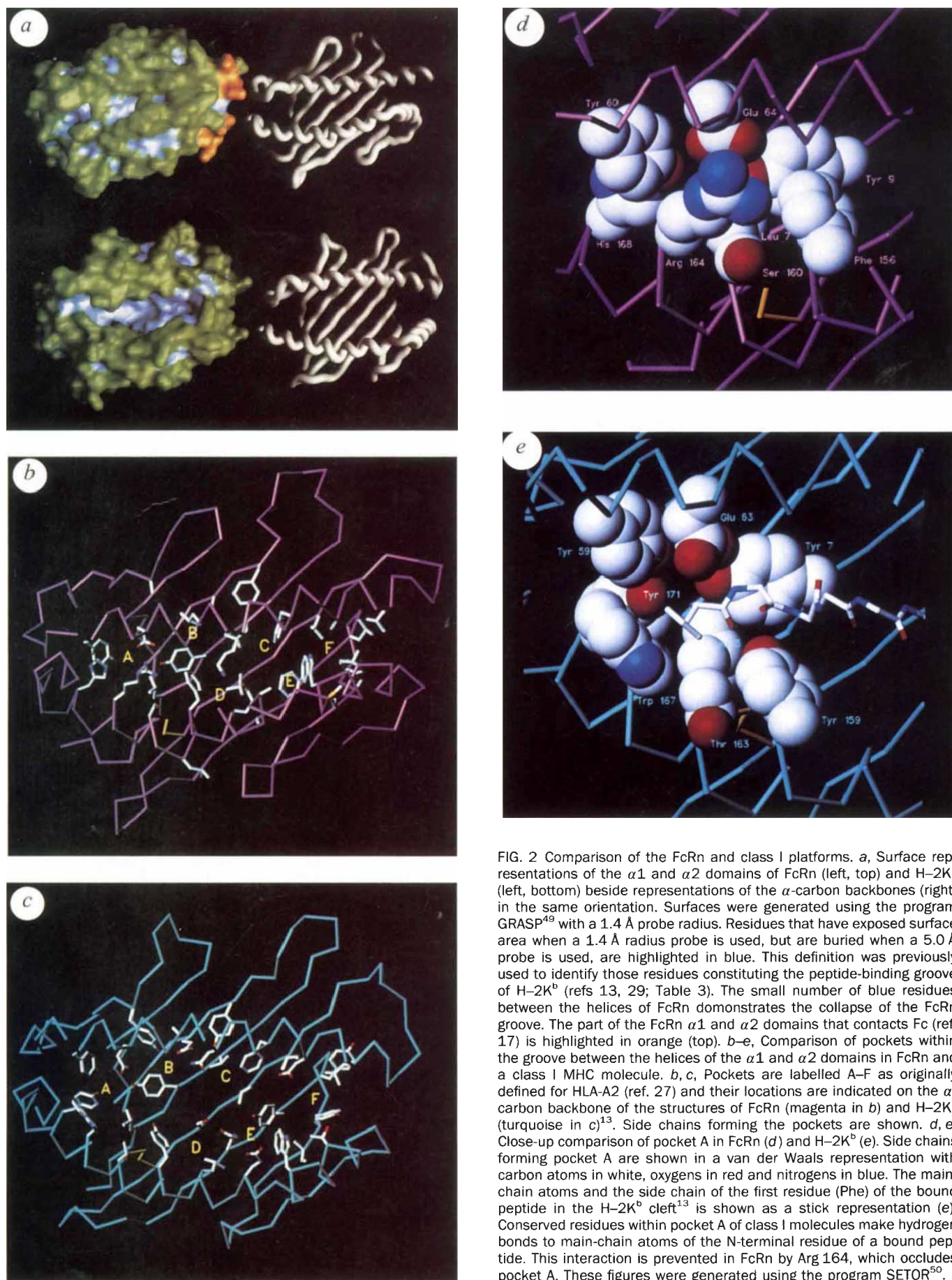


FIG. 2 Comparison of the FcRn and class I platforms. *a*, Surface representations of the $\alpha 1$ and $\alpha 2$ domains of FcRn (left, top) and H-2K^b (left, bottom) beside representations of the α -carbon backbones (right) in the same orientation. Surfaces were generated using the program GRASP⁴⁹ with a 1.4 Å probe radius. Residues that have exposed surface area when a 1.4 Å radius probe is used, but are buried when a 5.0 Å probe is used, are highlighted in blue. This definition was previously used to identify those residues constituting the peptide-binding groove of H-2K^b (refs 13, 29; Table 3). The small number of blue residues between the helices of FcRn demonstrates the collapse of the FcRn groove. The part of the FcRn $\alpha 1$ and $\alpha 2$ domains that contacts Fc (ref. 17) is highlighted in orange (top). *b–e*, Comparison of pockets within the groove between the helices of the $\alpha 1$ and $\alpha 2$ domains in FcRn and a class I MHC molecule. *b*, *c*, Pockets are labelled A–F as originally defined for HLA-A2 (ref. 27) and their locations are indicated on the α -carbon backbone of the structures of FcRn (magenta in *b*) and H-2K^b (turquoise in *c*)¹³. Side chains forming the pockets are shown. *d*, *e*, Close-up comparison of pocket A in FcRn (*d*) and H-2K^b (*e*). Side chains forming pocket A are shown in a van der Waals representation with carbon atoms in white, oxygens in red and nitrogens in blue. The main-chain atoms and the side chain of the first residue (Phe) of the bound peptide in the H-2K^b cleft¹³ is shown as a stick representation (*e*). Conserved residues within pocket A of class I molecules make hydrogen bonds to main-chain atoms of the N-terminal residue of a bound peptide. This interaction is prevented in FcRn by Arg 164, which occludes pocket A. These figures were generated using the program SETOR⁵⁰.

TABLE 3 Comparison of residues in peptide-binding groove of H-2K^b with those in FcRn

H-2K ^b	Pocket	FcRn	Pocket
Leu 5	A	Leu 7	buried
Tyr 7	A, B	Tyr 9	buried
Val 9	B, C	Leu 11	buried
Tyr 22	C	Phe 24	buried
Glu 24	B	Ala 26	buried
Tyr 45	B	Ala 45	buried
Tyr 59	A	Tyr 60	A
Glu 63	A, B	Glu 64	buried
Ala 67	B	Leu 68	buried
Asn 70	B, C	Lys 71	B, C
Phe 74	C	Phe 75	C
Asp 77	F	Ala 78	buried
Leu 81	F	Leu 82	buried
Ile 95	F	Leu 92	buried
Val 97	C	Gly 94	buried
Ser 99	C, D	Leu 96	buried
Gln 114	C, D, E	Ala 110	buried
Tyr 116	C, E, F	Phe 112	buried
Tyr 118	C, D, E	Leu 114	buried
Tyr 123	F	Phe 119	buried
Ile 124	F	Met 120	buried
Thr 143	F	Val 139	buried
Trp 147	E, F	Trp 143	E, F
Glu 152	E	Ala 149	E
Leu 156	D, E	Glu 153	D, E
Tyr 159	A, D	Phe 156	A, D
Tyr 171	A	His 168	buried

Surface areas were calculated using the coordinates of H-2K^b (ref. 13) (excluding peptide and water molecule coordinates) and the refined coordinates of FcRn (excluding water molecules) using X-PLOR¹⁹. The residues listed in column 1 were identified as part of the H-2K^b binding groove if they had exposed surface areas when a 1.4 Å radius probe is used, but were inaccessible when a 5.0 Å radius probe is used²⁹. Column 2 identifies the pocket or pockets to which the residue contributes. Column 3 lists the FcRn counterpart of the H-2K^b residue. The term 'buried' in column 4 indicates that the FcRn residue was not accessible when a 1.4 Å probe is used for the surface-area calculation. The remaining residues in column 4 can be considered to form parts of residual pockets remaining in the space between the two FcRn helices.

side of the $\alpha 1$ helix and with the loops joining the first and third β -strands.

Evolutionary implications

On the basis of analysis of minimum evolution trees, it has been suggested that the precursors of classical class I MHC proteins, the non-classical class I molecule CD1, and FcRn diverged around the time of divergence of reptiles and mammals⁷. The MHC peptide-binding groove could have been lost by FcRn evolving from a class I-like precursor, or acquired by class I molecules evolving from an FcRn-like precursor. However, because examination of the FcRn/Fc co-crystal structure¹⁷ shows that the FcRn counterpart of the peptide-binding groove is not used in Fc binding, it seems unlikely that the MHC fold shared by FcRn and class I molecules evolved for Fc receptor function.

In this context, the analysis of the differences between the class I peptide-binding groove and its counterpart in FcRn is relevant. The residue primarily responsible for the rigid body movement of the FcRn $\alpha 2$ helix and outermost β -strands is Pro 162, which introduces a kink in the $\alpha 2$ helix (Fig. 1d, e), resulting in closure of one side of the peptide-binding cleft. Classical human and murine class I molecules contain mainly valine at this position³⁰, but this residue in all CD1 sequences is a proline^{7,39}. In addition, there is a higher percentage of identity between the CD1 and FcRn $\alpha 2$ domains than between other domains of CD1 and their FcRn counterparts, suggesting that

this part of CD1 is the part that is most structurally similar to FcRn. This similarity may suggest that FcRn diverged early from class I molecules, at the time of differentiation into class I and CD1. Whether an FcRn-like structure in the $\alpha 2$ domain can be compatible with peptide binding remains open. It is possible that the FcRn counterpart of the MHC peptide binding groove plays some functional role in FcRn—perhaps the binding of an ion or small molecule at pH 7.5 that leads to a conformational change²³, allowing the release of bound IgG. Ultimately a comparison between this FcRn structure (solved at pH 6.5, the permissive pH for IgG binding) and a structure above pH 7.5 should facilitate a more complete understanding of the events involved in IgG binding and release. □

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as a three-terminal device with gain. In this sense, the analogy of the polymer network grid in the PGT with the grid in the vacuum-tube triode is genuine.

The current gain, G , is shown as a function of frequency in the inset to Fig. 3; $G = \Delta I_{AC} / \Delta I_{AC}$, where ΔI_G is the change in grid current and ΔI_{AC} is the resulting change in I_{AC} . An external a.c. signal (5 V peak-to-peak) was applied to the grid with $V_{AC} = -12$ V. At low frequencies $G \approx 4.5$, consistent with estimates based on the change in the d.c. characteristics at different V_G . On increasing the modulation frequency (f), G falls to one-half of the low-frequency value at $f \approx 400$ Hz, indicating a response time $\tau \approx 4 \times 10^{-4}$ s.

The high-frequency response is limited by the resistance/capacitance (RC) time constant. The input resistance to the grid is determined by the PANI-CSA network resistance; $R_n = \rho_n L / Ld = \rho_n / d$, where ρ_n is the resistivity of the network, L is the lateral dimension (assuming a square device) and d is the thickness of the device. The capacitance is determined by the thin-film ('capacitor') geometry, $C = \epsilon_s A / d = \epsilon_s L^2 / d$, where ϵ_s is the dielectric constant of the semiconductor. Thus $\tau_{RC} = RC =$

$\rho_n \epsilon_s (L/d)^2$. The measured values of R_n and C are consistent with the high-frequency cut-off shown in Fig. 3. The device area in this initial study was 0.2×0.6 cm. Size reduction would increase the RC-limited frequency response; by decreasing the lateral dimensions to 100 μm , $1/\tau_{RC} \approx 10^6 \text{ s}^{-1}$.

The theoretical limit for the high-frequency cut-off can be estimated. To obtain significant gain, carriers must move through the grid in a time comparable with the period of the a.c. signal. The carrier velocity is $2\mu V_G / d$, where μ is the carrier mobility. Thus, the μ -limited response time is $\tau_i \approx d^2 / 2\mu V_G$. With $V_G = 5$ V and $d = 2,500$ Å, $\tau_i \approx 4 \times 10^{-3} \mu$. For example, with $\mu \approx 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$, as reported for semiconducting polymers^{2,3}, $\tau_i \approx 4 \times 10^{-7}$ s. The short response time with such a small mobility is a direct result of the thin-film architecture of the polymer grid triode.

Higher gain can be achieved by improving the rectification ratios of the back-to-back diodes, by making the overall structure (and the component layers) thinner, by optimizing the concentration of PANI-CSA in the network for optimum transmission of carriers through the network grid, and by using a polymer semiconductor with a higher mobility. □

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Size-dependent catalytic activity of supported metal clusters

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BECAUSE catalysis by metals is a surface phenomenon, many technological catalysts contain small (typically nanometre-sized) supported metal particles with a large fraction of the atoms exposed¹. Many reactions, such as hydrocarbon hydrogenations, are structure-insensitive, proceeding at approximately the same rate on metal particles of various sizes provided that they are larger than about 1 nm and show bulk-like metallic behaviour¹. But it is not known whether the catalytic properties of metal particles become size-dependent as the particles become so small that they are no longer metallic in character. Here we investigate the catalytic behaviour of precisely defined clusters of just four and six iridium atoms on solid supports. We find that the Ir_4 and Ir_6 clusters differ in catalytic activity both from each other and from metallic Ir particles. This raises the possibility of tailoring the catalytic behaviour of metal clusters by controlling the cluster size.

Investigations of gas-metal clusters demonstrate that their reactivities depend strongly on their structures. Reactions of D_2 with Fe, Ni, Pd and Pt clusters show striking patterns of both reaction rates and coverage of clusters with D ligands depending on the metal, and the charge and number of atoms in the cluster^{2,3}. The reactivity of gas-phase Co_4^+ for dehydrogenation of cyclohexane to give benzene⁴ is markedly different from that

of Co clusters with only one atom more or one atom less, and the reactivity of Fe_4^+ for formation of benzene from smaller hydrocarbons is similarly unique⁵.

The fascination with molecular metal clusters⁶ motivated many investigations of their catalytic properties, but few examples of molecular cluster catalysis and no industrial applications have emerged^{7,8}. A decisive limitation is the lack of stability of most ligand-stabilized clusters. But metal clusters dispersed on solid supports have been thought to exist among the complex mixtures of structures in industrial supported metal catalysts; recently, clusters of (on average) 5–6 Pt atoms in the pores of zeolite LTL have been identified by extended X-ray absorption fine structure (EXAFS) spectroscopy⁹ and applied industrially as catalysts for selective reforming of naphtha to give aromatics^{10,11}. Thus the metal clusters of most importance as catalysts are robust supported clusters and not molecular clusters.

We have investigated the catalytic activities of Ir_4 and Ir_6 clusters on porous metal oxide supports for structure-insensitive hydrocarbon hydrogenation reactions. The robustness of the Ir_4 cluster frame has been shown by electrospray mass spectrometry experiments with a salt of $[\text{HIr}_4(\text{CO})_{11}]^-$, which underwent clean decarbonylation (removal of CO ligands) to give products with compositions $\text{HIr}_4(\text{CO})_{11-x}$ ($x = 1, 2, \dots, 11$) without fragmentation of the tetrahedral Ir_4 frame. Precursors of the supported catalysts were prepared by adsorption of $[\text{Ir}_4(\text{CO})_{12}]$ on supports (MgO and $\gamma\text{-Al}_2\text{O}_3$), and by formation of $[\text{Ir}_6(\text{CO})_{16}]$ in supercages of the molecular-sieve zeolite NaY by carbonylation of sorbed $[\text{Ir}(\text{CO})_2(\text{acac})]$ (acac, acetylacetonate). The resultant metal carbonyl clusters, $[\text{HIr}_4(\text{CO})_{11}]^-$ and $[\text{Ir}_6(\text{CO})_{15}]^{2-}$ on MgO (refs 12, 13), $[\text{Ir}_4(\text{CO})_{12}]$ on $\gamma\text{-Al}_2\text{O}_3$ (ref. 14) and $[\text{Ir}_6(\text{CO})_{15}]$ in zeolite NaY (ref. 15), were treated in He at 573 K, leading to complete decarbonylation, as shown by infrared and EXAFS spectra.

EXAFS spectroscopy shows that the first-shell Ir–Ir coordination numbers characterizing the fresh and used MgO -supported catalysts made from $[\text{Ir}_4(\text{CO})_{12}]$ are indistinguishable from 3, the value for a tetrahedron, as in $[\text{Ir}_4(\text{CO})_{12}]$ and $[\text{HIr}_4(\text{CO})_{11}]^-$

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TABLE 1 EXAFS results characterizing the precursors and catalysts

Sample number	Catalyst precursor	Support	Treatment	$N_{\text{Ir-Ir}}^*$, fresh catalyst	$R^\dagger$, fresh catalyst (nm)	$N_{\text{Ir-Ir}}^*$, used catalyst	$R^\dagger$, used catalyst (nm)	Reference
1	$[\text{Hlr}_4(\text{CO})_{11}]^-$	MgO	None	3.2	0.271			12, 13, this work
2	$[\text{Ir}_4(\text{CO})_{12}]$	$\gamma\text{-Al}_2\text{O}_3$	Decarbonylation and catalysis of toluene hydrogenation	2.9	0.269	3.2	0.268	14, this work
3	$[\text{Hlr}_4(\text{CO})_{11}]^-$	MgO	Decarbonylation and catalysis of toluene hydrogenation	3.2	0.271	2.8	0.269	12, 13, this work
4	$[\text{Ir}_6(\text{CO})_{16}]$	Zeolite NaY	Decarbonylation and catalysis of toluene hydrogenation			3.9	0.270	This work
5	$[\text{Ir}_6(\text{CO})_{15}]^{2-}$	MgO	Decarbonylation and catalysis of toluene hydrogenation			4.1	0.269	This work
6	$[\text{Hlr}_4(\text{CO})_{11}]^-$	MgO	Decarbonylation, treatment in H_2 at 573 K, and catalysis of toluene hydrogenation	3.1	0.270	3.1	0.270	This work
7	$[\text{Ir}_6(\text{CO})_{15}]^{2-}$	MgO	Decarbonylation, treatment in H_2 at 573 K, and catalysis of toluene hydrogenation	3.8	0.269	3.7	0.270	12, 13, this work
8	$[\text{Ir}_4(\text{CO})_{12}]$	$\gamma\text{-Al}_2\text{O}_3$	Decarbonylation, treatment in H_2 at 573 K, and catalysis of toluene hydrogenation			5.4	0.267	This work
9	$[\text{Ir}_6(\text{CO})_{16}]$	Zeolite NaY	Decarbonylation, treatment in H_2 at 573 K, and catalysis of toluene hydrogenation	3.6	0.271	4.1	0.269	15, this work
10	$[\text{Hlr}_4(\text{CO})_{11}]^-$	MgO	Decarbonylation, treatment in H_2 at 573 K, and catalysis of cyclohexene hydrogenation			3.3	0.271	This work

* First-shell Ir-Ir coordination number; estimated experimental error, $\pm 20\%$.

† Average Ir-Ir distance in clusters; estimated experimental error, $\pm 2\%$.

(Table 1, samples 3, 6 and 10). The decarbonylated clusters retained this metal frame. EXAFS shows that the decarbonylated Ir_6 clusters had metal frames indistinguishable from the octahedra of the precursor hexairidium carbonyls, indicated by the coordination number of approximately 4 (Table 1, samples 4, 5, 7 and 9).

Catalytic activities (Table 2) are reported as rates of reaction per exposed iridium atom (turnover frequencies); for Ir_4 and Ir_6 catalysts, these are rates per total Ir atom. The rates for conventional catalysts consisting of aggregates of metallic Ir or Pt on supports are reported per surface metal atom, determined by H_2 chemisorption. The conventional catalysts are ill-defined structurally, consisting of nonuniform particles of metal with (on average) about 50–70 metal atoms per particle.

Iridium carbonyl clusters $[\text{Hlr}_4(\text{CO})_{11}]^-$ on MgO are catalytically inactive for toluene hydrogenation; activity increased monotonically with fractional removal of CO ligands, indicated by infrared spectroscopy. The orders of reaction in toluene and in H_2 are approximately 0 and 1, respectively, for all the catalysts. The decarbonylated Ir clusters are markedly less active than the metallic aggregates on MgO (Table 2). Ir_6 clusters are several times less active than Ir_4 clusters. The effect of the support on the activities of the decarbonylated clusters is small (Table 2, samples 2 and 3; samples 4 and 5).

Pretreatment in H_2 at 573 K did not lead to a significant change in the metal framework structures of MgO-supported Ir_4 and Ir_6 , as shown by EXAFS, but it did lead to a decrease in their activities for toluene hydrogenation (Table 2, samples 3 and 6; samples 5 and 7). The results suggest that hydrogen

bonded strongly to the clusters and inhibited catalysis. The clusters chemisorb several times less hydrogen per exposed atom than supported Ir particles, and these adsorb even less than transition-metal clusters in the gas phase^{2,3}. Temperature-programmed desorption of chemisorbed hydrogen from the supported clusters indicates that some hydrogen is much more tightly bound than hydrogen on metallic particles.

However, the effect of hydrogen illustrated by these data is not the same as that observed for Ir_6 in NaY zeolite. Hydrogen pretreatment of this catalyst led to a two-fold increase in its activity, without a change in the metal framework structure. The data suggest that the different supports lead to different effects of hydrogen treatment. Additional evidence of support effects is shown by the data for Ir_4 supported on $\gamma\text{-Al}_2\text{O}_3$; following treatment in H_2 at 573 K, the catalytic activity (per total Ir atom) increased ten-fold (Table 2, samples 2 and 8). The increase is explained at least in part by the EXAFS result for the used catalyst showing that the Ir had aggregated to give clusters of roughly 20 atoms each, on average (Table 1, samples 2 and 8). Thus the support significantly affects the stability of the clusters, whereas it affects their intrinsic catalytic activity relatively little.

The low catalytic activity of Ir_4 relative to that of aggregated (metallic) Ir correlates with the capacities of these structures for chemisorption of H_2 . Although the clusters catalyse at least two of the same reactions as metallic Ir, their catalytic character is different. The clusters are metal-like but not metallic. The concept of structure insensitivity is limited to catalysis by metallic surfaces. The supported clusters are thus regarded as quasi-molecular, with the support providing part of a ligand shell,

TABLE 2 Catalytic activities of supported metal clusters and supported metallic particles

Sample number	Catalyst*	Support	Catalytic reaction†	H ₂ treatment temperature of catalyst K	TOF‡ (10 ⁻³ s ⁻¹)	E _{act} § (kJ mol ⁻¹)	Reference
1	[Hlr ₄ (CO) ₁₁] ⁻	MgO	Toluene hydrogenation	No treatment	0.00	—	This work
2	Ir ₄	γ-Al ₂ O ₃	Toluene hydrogenation	No H ₂ treatment	0.94	48	This work
3	Ir ₄	MgO	Toluene hydrogenation	No H ₂ treatment	0.63	58	This work
4	Ir ₆	NaY zeolite	Toluene hydrogenation	No H ₂ treatment	0.25	47	This work
5	Ir ₆	MgO	Toluene hydrogenation	No H ₂ treatment	0.23	56	This work
6	Ir ₄	MgO	Toluene hydrogenation	573	0.17	53	This work
7	Ir ₆	MgO	Toluene hydrogenation	573	0.03	53	This work
8	Aggregates of ~20 atoms each, on average, formed from Ir ₄	γ-Al ₂ O ₃	Toluene hydrogenation	573	9.9	42	This work
9	Ir ₆	NaY zeolite	Toluene hydrogenation	573	0.52	41	This work
10	Ir ₄	MgO	Cyclohexene hydrogenation	573	18	—	This work
11	Ir _{agg}	MgO	Toluene hydrogenation	723	2.0	55	This work
12	Pt _{agg}	γ-Al ₂ O ₃	Toluene hydrogenation	723	25	49	This work
13	Pt _{agg}	γ-Al ₂ O ₃	Toluene hydrogenation	723	27	51	16
14	Pt _{agg}	γ-Al ₂ O ₃	Cyclohexene hydrogenation	673	440	—	This work
15	Pt _{agg}	γ-Al ₂ O ₃	Cyclohexene hydrogenation¶	673	861	—	17

* The subscript agg refers to non-uniform metallic aggregates on the support. The average size of Ir_{agg} is ~1.3 nm and that of the Pt aggregates is ~1.1 nm, as determined by hydrogen chemisorption. The Pt_{agg} catalyst used for toluene hydrogenation was provided by M. A. Vannice; that used for cyclohexene hydrogenation was a commercial sample.

† Reaction conditions: toluene hydrogenation carried out with vapour-phase reactants in a once-through plug-flow reactor operated at atmospheric pressure and temperatures in the range of 333–373 K; cyclohexene hydrogenation carried out in this work with liquid-phase cyclohexene in n-hexane solvent saturated with H₂ flowing through the stirred reactor, which was held at 298 K and atmospheric pressure.

‡ Turnover frequency for toluene hydrogenation at 333 K and for cyclohexene hydrogenation at 298 K.

§ Apparent activation energy, determined from the dependence of turnover frequency on absolute temperature.

|| In contrast to the activities of the other catalysts, the activity of this catalyst is expressed per total Ir atom to facilitate the comparison with the results for sample 2. The comparison shows that the activity, even per total Ir atom, increased as a result of aggregation of the Ir caused by pretreatment of the sample in hydrogen.

¶ In the work of ref. 17, a batch reactor was used at 307 K with H₂ at atmospheric pressure; thus the data provide a close but not exact comparison with the data of this work.

which affects the activity as ligands affect the activities of molecular metal-cluster catalysts⁷.

The MgO-supported Ir₄ and Ir₆ clusters are stable catalysts, as shown both by the lack of significant changes in their activities in steady-state operation in a flow reactor for days and by EXAFS results indicating retention of the metal framework structures during catalysis (Table 1). Thus supported metal-cluster catalysts are robust enough for practical application. The size dependence of the catalytic properties of the supported clusters is consistent with the observations of unique reactivities of size-selected gas-phase metal clusters. Our results suggest that it may be fruitful to search for reactions for which supported metal clusters have catalytic properties superior to those of conventional supported metals. □

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Indirect influence of ozone depletion on climate forcing by clouds

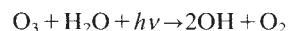
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CHEMICAL depletion of ozone in the lower stratosphere decreases the direct radiative forcing from greenhouse gases in the atmosphere¹. Here we show that ozone depletion may also exert an indirect effect on radiative forcing via its effect on the oxidation state of the atmosphere. Hydroxyl (OH) radicals in the troposphere are produced by photodissociation of tropospheric ozone in the presence of water vapour, and this process is enhanced if the absorption of ultraviolet radiation by the overlying stratospheric ozone column decreases. As OH oxidizes SO₂ to sulphuric acid, which then forms cloud condensation nuclei², variations in tropospheric OH concentration can influence cloud albedo. We use a global two-dimensional model forced by observed changes in stratospheric ozone to calculate the consequent changes in production of sulphuric acid over the past decade, and thus to estimate the effect on cloud albedo. We find that this indirect effect of ozone depletion may decrease radiative forcing (via increased cloud reflectivity) by at least as much as the direct effect.

The hydroxyl radical is primarily produced by the photodissociation of ozone (O₃) by radiation of wavelength near 310 nm in the presence of water vapour.



OH can then initiate the oxidation of SO_2 to sulphuric acid. The production rate, R , of sulphuric acid is thought to be a critical component in the nucleation and condensational growth of aerosol particles to sizes large enough to act as cloud condensation nuclei (CCN)³⁻⁵. Previous work^{3,4} has focused on possible increases in R due to increases in anthropogenic or natural SO_2 . But the production rate of sulphuric acid is also proportional to OH concentration, which is in turn partially governed by the overhead ozone column amount via the photolysis rate of O_3 . Here we attempt to assess the changes in sulphuric acid production rate resulting from stratospheric O_3 depletion.

In this study we use a global two-dimensional (latitude-height) model⁶⁻¹⁰. One run uses the 1989-90 total ozone mapping spectrometer (TOMS) data and the other the 1979-80 TOMS data. Gas-phase oxidation by OH is only a minor sink (less than 10%; ref. 11) for SO_2 . Figure 1 shows model calculations of the annual mean sulphuric acid production rate as calculated by the model for 1989-90. Maximum production occurs in the tropics (largest OH concentrations) and in the Northern Hemisphere mid-latitudes (high anthropogenic SO_2). R is small at high latitudes because of low OH concentrations, and decreases with altitude because of decreasing SO_2 . The decrease with altitude may not be realistic, because the model lacks a detailed representation of convective transport.

Figure 2 shows the percentage difference in the 2-year mean calculated OH concentration for 1989-90 relative to 1979-80. For the period 1979-90, global average O_3 decreased by ~3.5% (ref. 13). The decrease in stratospheric O_3 led to an increase in the ultraviolet flux reaching the troposphere¹⁴ and should have led to increased OH production by O_3 photolysis¹⁵. We calculate a global mean increase of ~3% for tropospheric OH. This increase is consistent with previous one-dimensional model^{15,16} and TOMS¹⁷ studies of OH sensitivity to O_3 column changes. OH increases mirror known reductions in the O_3 column. There is little change in the tropics, and large increases at high latitudes. The sensitivity of tropospheric O_3 photolysis and OH increases with O_3 optical depth¹⁷.

Figure 3 shows the calculated change in the mean sulphuric acid production rate for 1989-90 relative to 1979-80. Global mean production rate in the lower troposphere increases by 2.4%. Latitudinally, the increases in mean production rate mirror OH changes, with little change in the tropics but significant enhancements at higher latitudes. The enhancement of production rate decreases with altitude. In the upper troposphere, SO_2 decreases by up to 4%, because increased OH reduces the amount of SO_2 and dimethyl sulphide (DMS) reaching the higher altitudes.

There is now some suggestion that the eruption of Mount Pinatubo in June 1991 may have led to additional ozone depletion¹³. We have calculated the change in global mean sulphuric acid production rate for the years 1991-92 compared to 1979-80. R is found to increase by about 1% in the tropics because of a significant decrease in tropical O_3 column in 1991¹³. Changes at high latitudes are similar to those calculated for years 1989-90. Global mean sulphuric acid production rate increases by 2.9% while global OH is 3.6% larger. It should be noted that even larger O_3 depletions occurred in early 1993 leading to even larger changes in R in our model calculations.

What are the climate implications? If other factors controlling OH (for example, humidity, emissions of CH_4 and CO) had not changed, our model calculations suggest that R may have increased by 2.4% from 1979 to 1990. Correlations of CCN and levels of DMS^{18,19}, as well as aerosol modelling studies^{5,6} show that this increased production rate of sulphuric acid should translate into increased CCN and cloud droplet numbers^{18,20,21}. Following the same approach as previous work², we consider a change in the number of droplets for highly reflective marine stratus and stratocumulus clouds covering 30% of the Earth's surface. An increase in droplet number (keeping the same liquid-water content) is expected to decrease the effective drop radius

and increase the scattering of the solar flux. Charlson *et al.*² estimated a planetary solar albedo (denoted here by A_{GM}) sensitivity ($d A_{\text{GM}}/d \ln N$) to a change in droplet number (N) of these clouds as 0.02. The annual mean radiative forcing (ΔF_c) for an albedo perturbation can be evaluated by the following expression² $\Delta F_c = (1/4)F_T \Delta A_{\text{GM}}$, where F_T is the top-of-the-atmosphere flux ($1,370 \text{ W m}^{-2}$).

To equal the estimated¹ global direct forcing of -0.08 W m^{-2} by stratospheric O_3 depletion requires an increase of 0.00024 in the global mean albedo. This is equivalent to a 1.2% increase in the cloud droplet number. This forcing can also be compared to a forcing of $+0.5 \text{ W m}^{-2}$ by increased greenhouse gases over the last decade¹.

As in the previous studies^{2,5} the calculated indirect forcing is subject to a number of uncertainties. The global albedo sensitivity to droplet number is of unknown uncertainty⁵. Another key uncertainty is the relationship between the sulphuric acid production rate and the concentration of CCN. Aerosol modelling studies^{3,4} show that under conditions of efficient CCN production (low initial surface area characteristic of the free troposphere), the production sensitivity (the percentage increase in CCN per percentage increase in R) ranges from 1 to 4. A recent model study²² considering cloud processing of the sulphate size distribution suggests that the concentration of CCN is proportional to R .

There are no observations relating the concentration of CCN and the production rate of sulphuric acid (the source of new particles). However, several expressions relating the concentration of CCN to sulphur flux or mass have been derived from observations. Using linear and nonlinear expressions based on

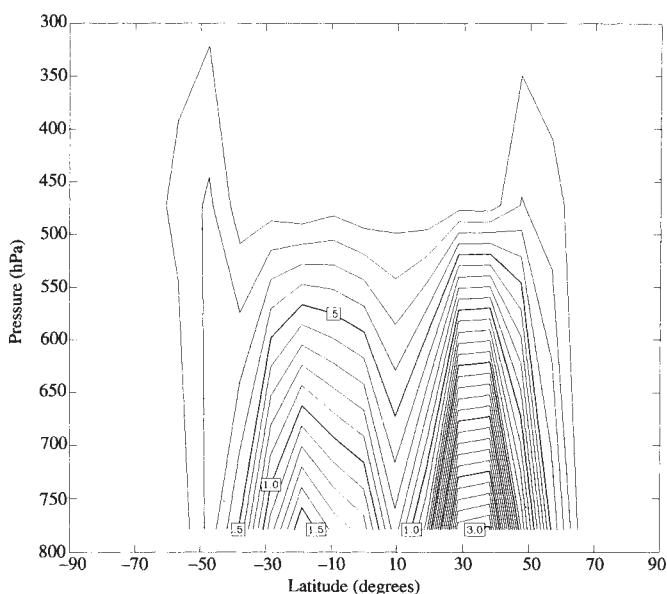


FIG. 1 The 2-year mean production rate of sulphuric acid (R , molecule $\text{cm}^{-3} \text{ s}^{-1}$) for 1989-90. Observed monthly mean O_3 columns from the total ozone mapping spectrometer (TOMS version 6.0) are used to force changes in the tropospheric ultraviolet flux. The flux is calculated using a two-stream method including scattering by clouds and aerosols⁹. The model includes latitudinally varying emissions⁹ of sulphur (total of 104 Tg S yr^{-1}) as SO_2 and DMS. DMS emissions are proportional to daylight hours to account for seasonal variation. For simplicity, we assume that DMS only reacts with OH and produces only SO_2 (ref. 10). Constant annual emissions of NO_x and CO are 30 Tg N yr^{-1} and 828 Tg C yr^{-1} respectively. First order total heterogeneous loss of SO_2 by dry/wet deposition and on marine aerosol is assumed²⁹ to be $5 \times 10^{-6} \text{ s}^{-1}$; loss of SO_2 in clouds is assumed to be rate-determined by the lifetime of the cloud^{11,30}.

DMS flux and CCN¹⁹, a 2.4% increase in the global mean DMS flux would increase the concentration of CCN by 1.2%. Another study²³ using nonlinear relationships finds for typical marine conditions, a 2.4% increase in mass gives an increase in cloud droplet number of only 0.5–1%. On the other hand, there are observations²⁴ of significant linear relationships; a 2.4% increase in H₂SO₄ or sulphate mass would increase the concentrations of CCN by 1.8% and 2.4% respectively. Overall, based on observations and model calculations, the CCN production sensitivity can be estimated to range from 0.2 to 4. The global 2.4% increase in R should therefore have translated into an increase in CCN ranging from 0.5 to 9.6%. This suggests that the radiative forcing of the CCN perturbation may have represented between 40 and 800% of the direct radiative forcing of O₃ in the last decade. The total (direct + indirect) climate forcing by O₃ depletion during the 1980s may therefore have been sufficient to offset the enhanced greenhouse warming. This indirect ozone–cloud effect is also about an order of magnitude larger than, for example, a reduction of 1.2% in methane caused by 1.2% increase in OH²⁵.

Furthermore, the global change in R is not uniform. Although the calculated change in the global mean rate of sulphuric acid production is 2.4%, increases several times larger are calculated at latitudes greater than 40° (Fig. 3). Coincidentally, the radiative short-wave forcing by clouds seems to peak at mid-latitudes^{5,26}. Comparing these estimates with previous studies² investigating the impact of anthropogenic SO₂ on CCN, it is worth noting that our estimated CCN perturbation is not local because O₃ depletion is widespread.

Limited observational support for our mechanism is provided by a large trend (+9.6% yr⁻¹) in condensation nuclei number detected in Antarctica²⁷. In this clean region, which should show the clearest evidence for our proposed mechanism, we calculate an annual mean increase in R of 20–30% over the last decade (Fig. 3). Aerosol model⁴ calculations show that this increase in R could indeed increase the number of condensation nuclei by over 100%. But unlike the number of condensation nuclei, the measured size distribution may not be consistent with this notion.

We also studied model sensitivity to the background atmosphere by varying DMS, SO₂, NO_x (NO + NO₂) and CO emis-

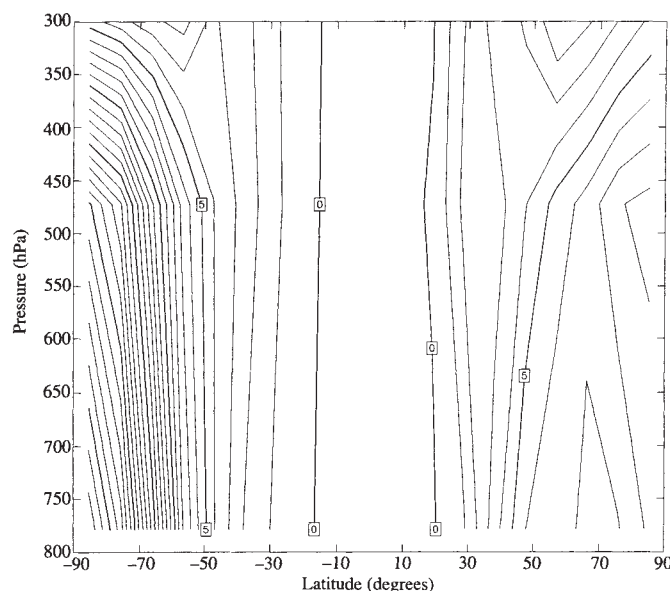


FIG. 2 Percentage change of OH for the 2-year mean 1989–90 compared to 1979–80.

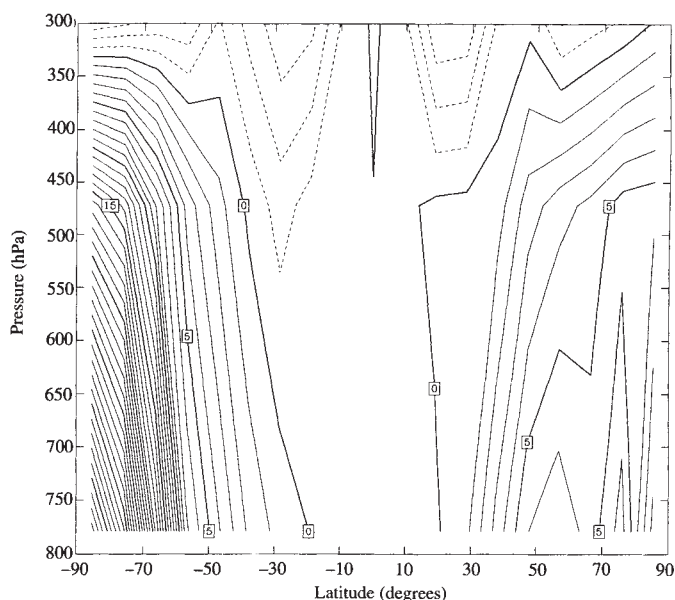


FIG. 3 Percentage change of sulphuric acid production rate for the 2-year mean 1989–90 compared to 1979–80. Note that in the south polar region, although the percentage change is large the absolute values remain rather small because SO₂ is small here.

sions and heterogeneous SO₂ loss rates by a factor of 2. We found that the absolute changes in R were strongly affected, but not the percentage changes. The global mean percentage change always exceeds 2.3%. This lack of sensitivity is not totally surprising. Overall, SO₂ is weakly dependent on OH and therefore percentage changes in R reflect essentially percentage changes in OH.

Whether OH (and therefore CCN production) has increased over the last decade is unknown. Here we calculate an increase in global tropospheric OH of about 3% due to O₃ depletion. It is possible that such changes in OH levels may have been masked by changes of CH₄, CO and NO_x during the last decade. Nevertheless, the proposed stratospheric ozone/cloud mechanism should in principle operate and force climate. The concentration of stratospheric ozone is affected by a number of other processes such as the solar cycle, the quasi-biennial oscillation and volcanic eruptions, which may therefore also force the climate via CCN production. Finally, climate models²⁸ predict that for a doubling of CO₂, tropospheric water vapour would increase by ~30%, which we estimate may increase OH and CCN production by 15%. This would translate into a forcing of ~1 Wm⁻². Despite the uncertainties of our estimates it is clear that the potential effect on climate of perturbing tropospheric OH, for example by changing stratospheric ozone levels, may be much larger than previously thought. More studies are clearly required. □

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Metastability of enstatite in deep subducting lithosphere

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OLIVINE and (Mg,Fe)SiO₃ pyroxene, the most abundant minerals in the Earth's upper mantle, are believed to transform to high-pressure phases at ~400 km depth^{1–5}. The possible metastable persistence of olivine to greater depths in some subduction zones—with consequences for the origin of deep-focus earthquakes and the dynamics of subduction—has been discussed extensively^{6–14}, but the role of other mantle minerals has not been considered. We report here an experimental study of the kinetic behaviour of the magnesian pyroxene enstatite (MgSiO₃) at upper-mantle conditions. We find that, whereas forsterite (Mg-olivine, Mg₂SiO₄) transforms rapidly to β -phase at 1,200 °C and 16 GPa, the transformation of enstatite to β -phase plus stishovite on the same time-scale requires much higher temperatures. At lower temperatures, enstatite transforms directly to the ilmenite structure, but only at pressures greater than 20 GPa. Enstatite should therefore persist metastably to greater depths than olivine in subduction zones, transforming directly to the ilmenite structure. The enstatite-ilmenite transformation is accompanied by a large decrease in volume, which should increase the stresses in subducting slabs, change the buoyancy forces that drive subduction, and might also contribute to the origin of deep-focus earthquakes.

At upper-mantle pressures, (Mg,Fe)SiO₃ enstatite constitutes on average ~25 vol% of subducting lithosphere and locally, within pyroxenite layers, the proportion can be as high as 100 vol% (ref. 15). Therefore the transformation kinetics of this phase are also likely to be important for subduction dynamics and mechanical behaviour of subducting slabs. In subduction zones, if equilibrium were maintained, enstatite would be expected to react out by a depth of ~450 km, becoming incorporated into a garnet solid solution¹. But during metamorphism of crustal rocks at high pressure (≥ 2 GPa and 600–750 °C), rates of intergranular diffusion are extremely slow when pervasive deformation and associated fluids are absent. The result is that individual mineral grains react in chemical isolation from neighbouring grains and form domains of local equilibrium^{16–18}. Based on these observations, it is likely that reaction between garnet and enstatite will be inhibited by slow rates of both intercrystalline and intracrystalline diffusion at low temperatures (500–600 °C) within subducting slabs. We therefore predict that the near-isochemical transformation of enstatite grains to β -(Mg,Fe)₂SiO₄ + stishovite (or to other high-pressure phases, as discussed below) occurs preferentially within subducting lithosphere. Note that this reaction would actually involve high-clino-

enstatite, which is the stable polymorph of (Mg,Fe)SiO₃ at pressures above ~7 GPa (ref. 19).

In contrast to olivine, the mechanisms and kinetics of the transformation of enstatite at high pressure have previously not been studied. Here we report results of experiments in which hot-pressed forsterite + enstatite aggregates were reacted in the stability fields of β -Mg₂SiO₄ + stishovite, γ -Mg₂SiO₄ + stishovite, and MgSiO₃ ilmenite, at 16–21 GPa in the temperature range 1,000–1,650 °C.

Multi-anvil high-pressure experiments²⁰ were performed using a powdered mixture of Mg₂SiO₄ forsterite (60 vol%) and MgSiO₃ enstatite (40 vol%) as the starting material. Each experiment consisted of two stages: (1) hot-pressing in the forsterite + high-clinoenstatite stability field at 12 GPa and 1,350 °C for 3.5 h, and (2) reacting the sample in the stability field of high-pressure phases (Fig. 1). The initial hot-pressing (annealing) stage, which is necessary to achieve an equilibrium microstructure in the starting material²¹, resulted in forsterite + clinoenstatite aggregates with low dislocation densities and a grain size of up to 15 μ m (Fig. 2a).

Following hot-pressing, the temperature was first decreased to 600 °C, then the pressure was increased to the desired value (16–21 GPa). Finally the temperature was increased to a value in the range 1,000–1,650 °C and maintained there for times varying from 45 min to 30 h to produce transformation to the high-pressure phases (Fig. 1). Following the experiments, each sample was first prepared as a thin section for examination by optical microscopy and X-ray microdiffraction, and was subsequently ion milled for examination by transmission electron microscopy (TEM).

The results of the experiments are summarized in Table 1. In short duration experiments at relatively low pressure (16 GPa) the forsterite partially transforms to β -Mg₂SiO₄ by a mechanism

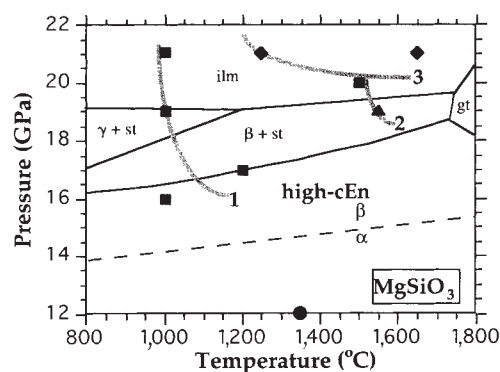


FIG. 1 MgSiO₃ phase diagram (after ref. 34) showing the conditions of the high-pressure experiments. The Mg₂SiO₄ α - β phase boundary is also shown (dashed line). Abbreviations are defined in Table 1. Circle, conditions used for hot pressing the forsterite + enstatite starting material; squares, no transformation of enstatite; triangle, very minor transformation of enstatite to β -phase + stishovite; diamonds, complete transformation of enstatite to ilmenite. The three curved shaded lines are semi-schematic kinetic boundaries showing approximate P - T conditions required for (1) the transformation of forsterite to β - or γ -phase in <1 h, (2) the initiation of the enstatite to β -phase + stishovite transformation after 30 h, and (3) complete transformation of enstatite to ilmenite after ~3 h. These curves emphasize semiquantitatively the difference in kinetics of the respective transformations of forsterite and enstatite. Note that on a geological timescale these kinetic boundaries move to considerably lower temperatures—it is estimated, for example, that ~600 °C is required for the (Mg,Fe)₂SiO₄ α - γ transformation to occur in subducting slabs¹⁴. Discrepancies between observed phases (Table 1) and this phase diagram may either be the result of uncertainties in the exact locations of the MgSiO₃ phase boundaries (refs 5, 33 and 34) or may result from the nucleation and growth of metastable phases²¹.

involving grain boundary nucleation and interface-controlled growth as described previously^{11,21–24}. At pressures ≥ 16 GPa and temperatures $\geq 1,200$ °C, the complete transformation of forsterite to β - and/or γ -phase is rapid and occurs in <1 h. In contrast, enstatite shows no evidence of reaction in any of the experiments at conditions up to 20 GPa and 1,500 °C and times up to 30 h, even though the enstatite = β -phase + stishovite equilibrium boundary was overstepped by pressures of 3–4 GPa. In experiments at $\geq 1,550$ °C and 19–21 GPa there was limited transformation of enstatite to β -phase + stishovite (Fig. 2b). At higher pressures (21 GPa) and temperatures of 1,250–1,650 °C, enstatite transforms rapidly (in ≤ 3.5 h) to MgSiO_3 ilmenite (Fig. 2c). The ilmenite sometimes shows evidence of subsequent transformation to γ -phase + stishovite (Table 1) which may be the result of a localized drop in pressure caused by the large negative volume change of the enstatite-ilmenite transformation.

The absence of enstatite transformation in the stability fields of β -phase + stishovite and γ -phase + stishovite, at temperatures below 1,550 °C, can be ascribed partly to the difficulty of nucleating stishovite, a phase which is structurally and chemically quite different from both olivine and enstatite. But even when stishovite nucleates, the rate of enstatite transformation is much slower than the polymorphic transformation of olivine to β - or γ -phase because diffusion distances are relatively large during eutectoid-type reactions involving two product phases (Fig. 2b). In contrast, the rate of the polymorphic enstatite to ilmenite transformation, for which a pressure of at least 21 GPa is required on an experimental timescale, is fast because long-range diffusion is not required.

Temperatures within rapidly subducting lithospheric slabs are estimated to be as low as 500–600 °C at depths of 400–500 km (refs 25–28). During crustal metamorphism under fluid-absent conditions in this temperature range, solid-solid reactions involving the nucleation of more than one product phase can be kinetically inhibited on a geological timescale²⁹. The transformations of enstatite to β -phase + stishovite and γ -phase + stishovite are also likely to be kinetically inhibited during subduction, particularly at temperatures which are $\sim 1,000$ °C lower than those required for these reactions to occur on a laboratory timescale. At low temperatures in subduction zones, it is likely that enstatite survives as a metastable phase through the β -phase + stishovite and γ -phase + stishovite stability fields and eventually transforms directly to the ilmenite structure.

It has been proposed that metastable olivine survives within a wedge-shaped region (bounded approximately by the 600 °C isotherm) to depths of 600 km or more in relatively cold subducting slabs^{6–14}. Our experimental results show that the transformation of enstatite to high-pressure phases (β -phase + stishovite, γ -phase + stishovite, or ilmenite) requires higher pressures and temperatures on a laboratory timescale than the transformation of olivine to the β - or γ -phases (Fig. 1). Assuming that this difference in kinetic behaviour persists on a geological timescale, a second wedge containing metastable enstatite, extending to a greater depth than the metastable olivine wedge, must exist in subduction zones. In this case the metastable enstatite wedge would be bounded by a higher-temperature isotherm and thus be thicker than the wedge containing metastable olivine.

The detailed mineralogical structure of subducting lithosphere and the location of phase boundaries affects the driving forces of subduction, stresses on the slab due to density contrasts with the adjacent mantle and internal stresses due to volume changes resulting from high-pressure phase transitions^{1,8,30–32}. The state of stress, in turn, affects the distribution of deep-focus earthquakes. The presence of a wedge of metastable olivine has a large effect on the internal stresses in the slab, as well as on the buoyancy forces which drive subduction, compared with the case where phase transformations occur close to equilibrium^{1,30–32}. The presence of a wedge of metastable enstatite must

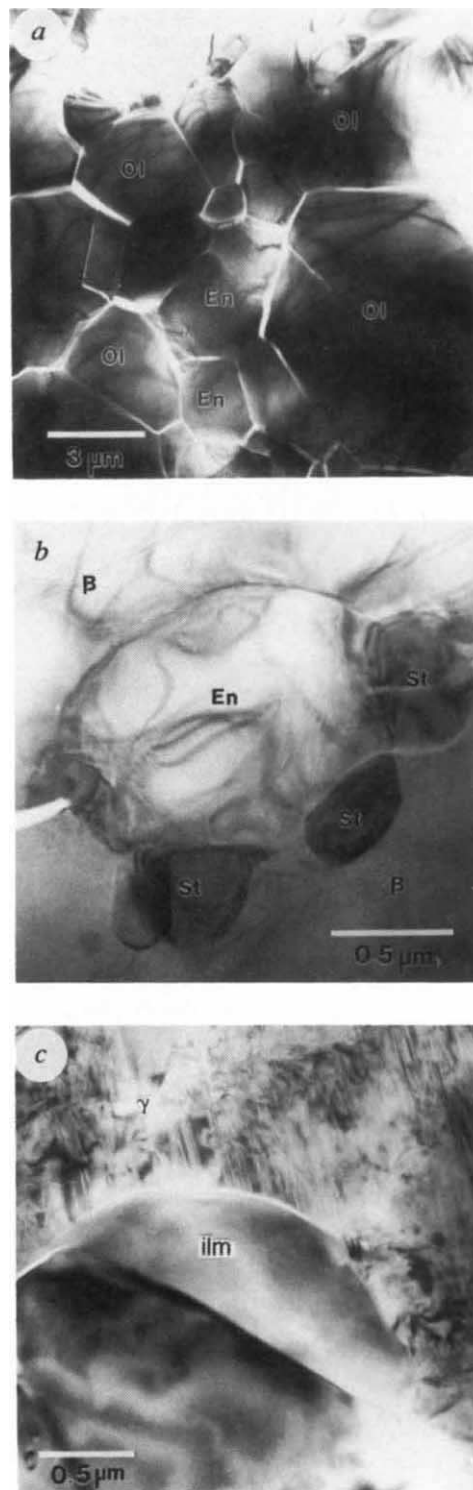


FIG. 2 a–c, TEM micrographs showing the results of hot pressing (a), partial transformation of enstatite to β - Mg_2SiO_4 + stishovite (b) and ilmenite which originated by transformation from enstatite (c). a, The hot-pressed sample consists of polygonal grains of Mg_2SiO_4 olivine (ol), 1–15 μm in diameter, together with smaller grains of clinoenstatite (En). The polygonal grain shapes and the relatively low defect densities represent the equilibrium microstructure that develops during hot pressing. b, Enstatite transforms to β - Mg_2SiO_4 (β) + stishovite (st) at temperatures $>1,500$ °C. β - Mg_2SiO_4 (β) in this sample originated mostly by the transformation of olivine. c, At lower temperatures (for example 1,250 °C) and higher pressures (≥ 21 GPa) enstatite transforms rapidly to ilmenite (ilm). γ - Mg_2SiO_4 (γ) originated by the transformation of olivine.

TABLE 1 Results of multi-anvil experiments

Run No.	Load (Tonne)	P* (GPa)	T† (°C)	t (min)	Results‡
H32	463	16	1,000	70	cEn + α + β
H48	463	16	1,000	360	cEn + α + β
H44	514	17	1,200	60	cEn + β
H52	657	19	1,000	60	cEn + β
H73	657	19	1,550	60	cEn + β (+st)
H83	743	20	1,500	1,800	cEn + β
H148	857	21	1,000	210	cEn + α + β + γ
H136	857	21	1,250	210	ilm + γ + st(+ β)
H129	857	21	1,650	45	ilm + β (cEn + st + gt)

* Experiments were performed using a 10-mm MgO octahedron as the pressure medium and WC (Toshiba F-grade) anvils with 5-mm truncations²⁰. The calibration of sample pressure as a function of load is from ref. 20 and the pressure uncertainty is $\pm 5\%$.

† Temperatures were measured with W3%Re/W25%Re thermocouples and were controlled to $\pm 2^\circ\text{C}$; deviations from the recorded value across the sample due to temperature gradients were $< \pm 30^\circ\text{C}$ (ref. 20).

‡ Abbreviations: α , α -Mg₂SiO₄ (forsterite); cEn, clinoenstatite; st, stishovite; ilm, MgSiO₃ ilmenite; β , β -Mg₂SiO₄; γ , γ -Mg₂SiO₄; gt, MgSiO₃ garnet. Minor phases (<5 vol%) are listed in parentheses.

also be considered when estimating stresses and buoyancy forces because, although enstatite is less abundant than olivine in subducting lithosphere, the clinoenstatite-ilmenite phase transformation involves a large volume decrease of $\sim 12\%$ (refs 19, 33).

In addition to having a large volume decrease, the polymorphic enstatite to ilmenite transformation is exothermic^{33,34} and therefore has the characteristics necessary for producing deep-focus earthquakes by the transformational faulting mechanism^{7,9}. Considering that enstatite is less abundant in oceanic lithosphere than olivine, there are two possible mechanisms for high-pressure faulting which could involve this transformation. (1) The enstatite to ilmenite transformation could occur at the same time as the olivine-spinel transition and thereby enhance the transformational faulting process. Although enstatite breakdown is kinetically more sluggish than the olivine-spinel transition, the latter transformation is exothermic and may therefore trigger the enstatite-ilmenite transformation through a localized temperature increase. (2) The transformation of enstatite to ilmenite might cause deep-focus earthquakes at depths below that at which the transformation of olivine to spinel (β - or γ -phase) is complete. In this case, a bimodal distribution of deep earthquakes might be observed below a depth of 400 km, caused by the two phase transformations occurring at different depths. The average enstatite content of subducting lithosphere is only $\sim 25\text{ vol}\%$ and it is uncertain whether this is sufficient for the transformational faulting mechanism to operate. We speculate that even isolated grains of enstatite transforming to ilmenite may cause faulting in a surrounding matrix of γ -(Mg,Fe)₂SiO₄ by initiating the type of plastic instability discussed by Hobbs and Ord³⁵. The observation that faulting (apparently caused by the ice I to ice II transition) occurs during deformation of water ice+ammonium dihydrate mixtures at high pressure, even when the content of water ice is only $\sim 10\%$, lends support to this hypothesis³⁶. □

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Propagation and amplification of tsunamis at coastal boundaries

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WHEN tsunamis hit a coastal area, they often cause substantial damage and loss of life at locations otherwise well protected from normal (even severe) wind-generated waves. An example of such a catastrophe took place on 12 December 1992, when an earthquake of magnitude $M_s = 7.5$ occurred in the eastern region of Flores Island, Indonesia^{1,2}. One of the areas hardest hit by the resulting tsunamis was Babi, a small coastal island; two villages on the island were completely destroyed, even though both were located in sheltered areas where wave conditions are normally calm. Here we describe the results of numerical and laboratory experiments that show how, unlike wind-generated waves, tsunamis are capable of penetrating into sheltered coastal areas without significantly losing their energy. For the case of Babi Island, the original tsunami wave was split into two, with one wave propagating around each side of the island. The two waves met in the sheltered region, and the subsequent amplification of wave amplitude resulted in the destructive flow onto the beach.

Babi Island has a conical shape, with a diameter of approximately 2 km and a summit elevation of 351 m (see Fig. 1); the surrounding waters are deep with a steep sea-bottom slope. The north shore faces the Flores Sea and has a wide coral reef. The destroyed villages (Kampungbaru and Pagaraman) were located

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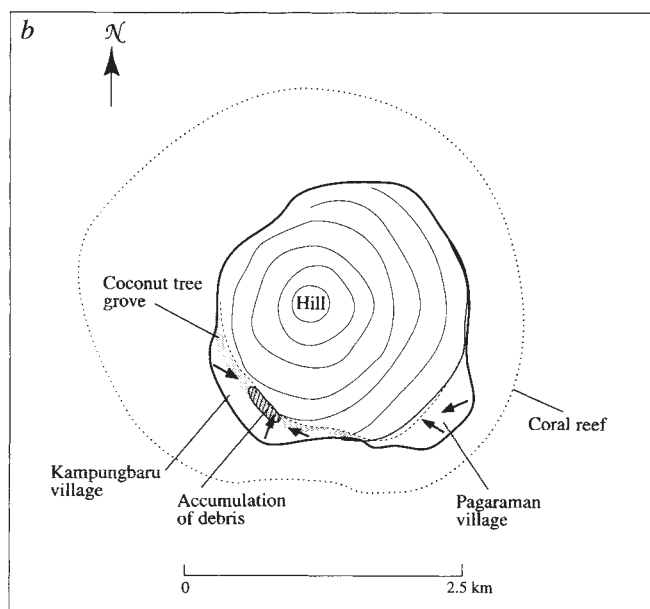
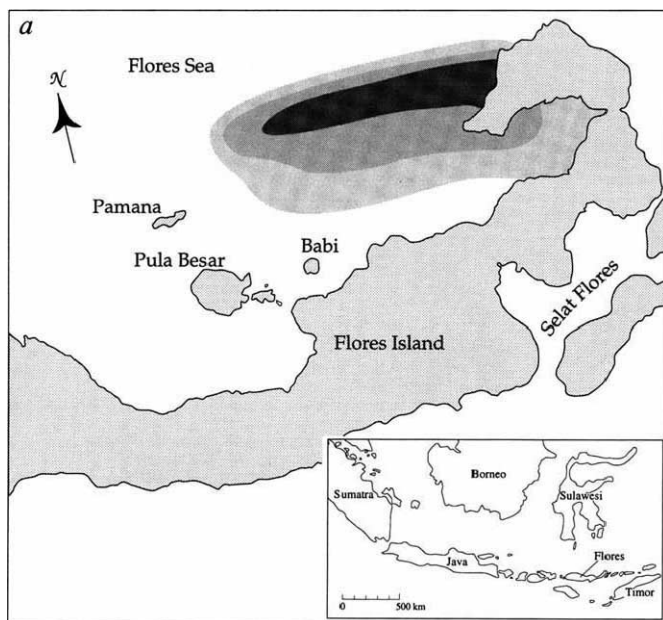


FIG. 1 *a*, Babi Island is located ~5 km offshore from Flores Island, Indonesia. Although detailed sea bottom sounding data are not available, the underwater slope from the island is steep: the 100-m-deep contour around the island is very close to the shore, one sounding in the narrow gap between Babi and Flores is 241 m deep, and the 860-m sounding point in the Flores Sea is 10 km from the island. The shaded areas in the Flores Sea, which represent 3-m, 2-m and 1-m contours, respectively, are estimated vertical tectonic displacement of sea floor where tsunamis were generated¹⁰. *b*, Details of Babi Island. Near the

middle of the south shore, there is a small tidal flat which separates Kampungbaru village (on the west side) from Pagaraman village (on the east side). Both villages were completely destroyed by the tsunamis. The tsunami run-up directions, indicated by solid arrow marks, were estimated from the directions of tree falls and debris accumulations (based on field notes taken by H.Y. and F. Imamura during the survey in 1992–93). The maximum tsunami run-up heights are 5.6 m in Pagaraman and 7.1 m in Kampungbaru, respectively.

on the island's south side, facing Flores Island, where a coral reef in front of the villages is much narrower than at the north shore. The distance between Babi and Flores islands does not provide enough fetch distance to produce any significant wind waves. Hence the village locations are in the "shadow" zone of the wave field, where waves in the Flores Sea are blocked and dissipated at the north side and the wave conditions are normally calm. These characteristics of natural protection make the south side of the island suitable for its small fishing villages. Tsunamis struck Babi Island from the north (Fig. 1*a*), so why were the villages completely destroyed?

Tsunamis are different from wind-generated waves. Wind waves in deep water contain most of their energy near the surface. The wave-induced water motion decays exponentially with depth; at a depth of even one-half of a wavelength, the water velocity is reduced to only 4% of the surface velocity. Hence even if the wave height is very large, the available wave energy flux is limited. On the other hand, tsunamis generated by tectonic motions contain the water flow in the entire water column. In an offshore location, the wavelength of a tsunami is usually long (of the order of tens to hundreds of kilometres) and its height is small, perhaps a few metres at most. Because the kinetic energy of a tsunami is evenly distributed throughout its entire depth, when it approaches the shore, its wave height increases by converting kinetic energy to potential energy. When the beach slope is steep, the conversion is especially efficient with little dissipation, meaning that more energy is available during run-up onto the beach.

It is known that a cone-shaped island is capable of trapping quasi-steady oscillatory waves around itself because of wave refraction, and that trapping occurs when the wavelength is comparable to the island dimension^{3,4}. Knowing this, we have conducted both laboratory⁵ and numerical⁶ experiments on tsunami run-up using a solitary wave as a tsunami model. A solitary

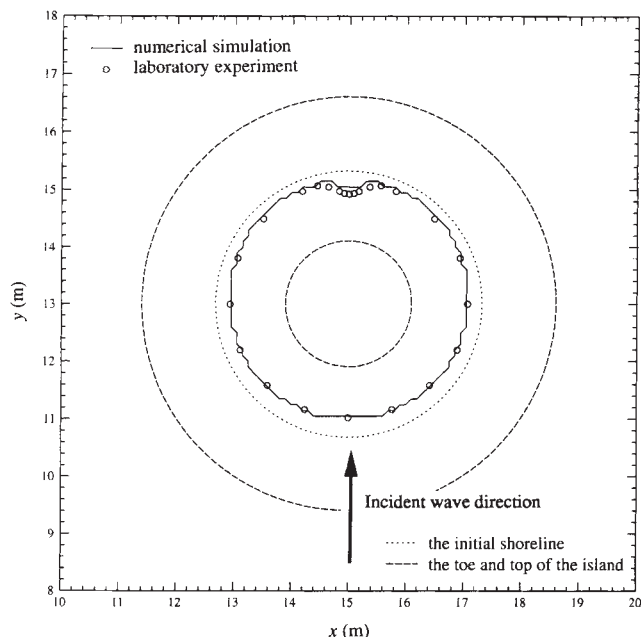


FIG. 2 The maximum water-run-up values around the island based on the laboratory experiment (circles) and numerical simulation (solid line). A solitary wave was generated at $y=0$ m with propagation direction from the bottom to the top of the figure. The ratio of wave amplitude to water depth at the place of generation is 0.1. The dashed lines denote the locations of the toe and top of the island, forming a 14° concentric beach slope, and the dotted line indicates the initial shoreline on the beach for the 0.32 m offshore water depth.

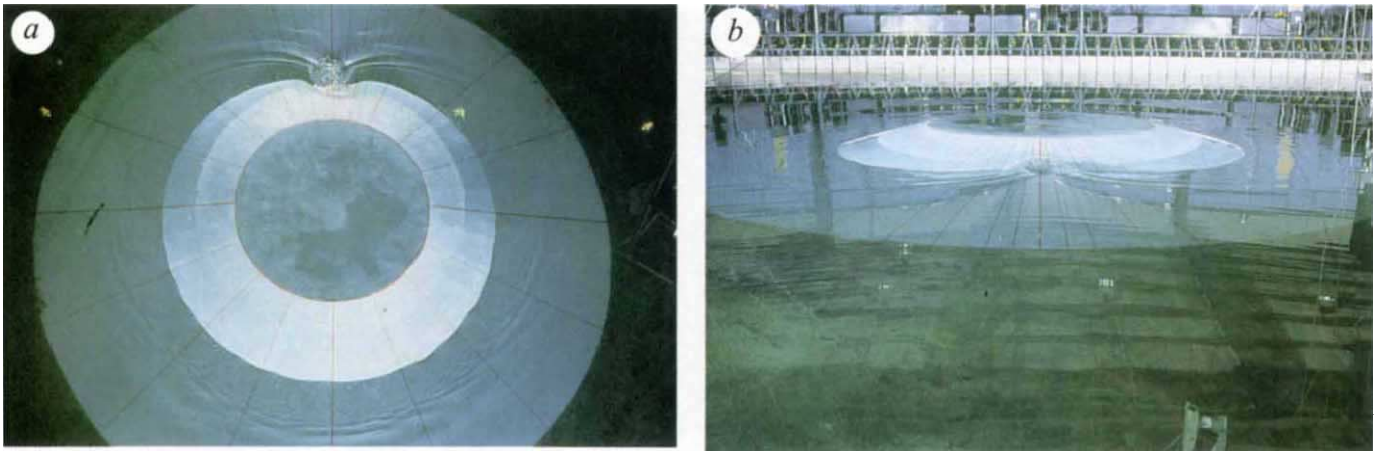


FIG. 3 Views of wave run-up onto the rear side of the cone-shaped island in the laboratory model. *a*, Top view; the wave struck the island from the bottom of the photograph. *b*, Wave paddles are visible beyond

the island. To generate a solitary wave in a precisely controlled manner, the hydraulic powered electro-servo system was used to set the vertical paddles in motion in the horizontal direction.

wave has a single positive crest, and is capable of maintaining its form during propagation⁷ by balancing weakly nonlinear and weakly dispersive effects. Because of its stable form and the fact that a leading wave of tsunamis often emerges as a solitary wave after a long period of propagation, it has become customary to use a solitary wave as a model of tsunami formation offshore⁸. The laboratory experiments were performed at the US Army Corps of Engineers, Waterway Experiment Station, Vicksburg, Mississippi. The wave basin used is 30 m wide, 25 m long and 0.32 m deep, with a model conical island 7.2 m in diameter with a 14° beach slope. A solitary wave was generated by the programmable wavemaker which spanned 27.4 m. The numerical model used is a staggered-grid finite-difference scheme based on the shallow-water wave approximation (assuming a hydrostatic pressure field of zero-viscosity fluid and a uniform flow field in the vertical direction); it simulated exactly the same conditions as the laboratory model.

Both laboratory experiments and numerical simulations demonstrate that, when the solitary wave hits the front side of the island, the wave is split into two. One wave propagates along one side of the island, while the other wave propagates along the other side. These propagating waves have their maximum wave amplitude along the shoreline with the crest perpendicular to the shoreline: these features resemble the characteristics of trapped waves. When these two waves collide behind the island (in the shadow zone) a substantial magnification of wave amplitude occurs near the shore, and subsequently results in significant flow (run-up) onto the beach surface. After the flow run-up, both laboratory experiments and numerical simulations clearly demonstrate formation of trapped waves around the island; one wave propagates back to the front of the island along one side of the island and the other propagates along the other, with amplitudes that decrease with time; these waves only exist around the island, consequently having finite energy, and are not related to the offshore wave field. The flow pattern and the run-up heights predicted numerically are in good agreement with the laboratory data (Fig. 2). Although the maximum run-up height occurs at the front of the island, the maximum value of the shoreline velocity is at the rear. The maximum run-up velocity at the rear of the island is 3.0 times that at the front, while the maximum run-up height at the front of the island is 1.7 times that at the rear. This is because the run-up processes are different at the front and rear faces of the island; the gradual run-up at the front and the sudden run-up at the rear. The latter is shown in Fig. 3. The laboratory results and the numerical simulations both show a flow convergence and run-up onto the beach behind

the island that are similar to the flow pattern on Babi inferred from our survey (Fig. 1*b*). According to field survey notes (H.Y. and F. Imamura) there are signs that suggest strongly that flow convergence and strong run-up occurred at Kampungbaru. The complete destruction and locations of debris accumulation from the village cannot be explained otherwise; all the debris was dumped by the tsunamis into a dense grove of coconut trees in the foothills. On the other hand, the flow pattern in Pagaramon village is generally from east to west, and the debris was scattered rather than accumulated in one location. This type of flow convergence would not occur if the wavelength were much shorter or longer than the island dimension. Shorter waves would be refracted completely and dissipate their energy by breaking on the beach, so no significant wave energy would be available in the shadow zone; the flow associated with longer waves would simply cause gradual increase and decrease of water level around the island without the formation of trapped waves.

Our laboratory and numerical simulations of tsunami run-up on the cone-shaped island provide a reason why the villages in Babi were totally destroyed by the tsunamis. They also underscore some important differences between tsunamis and wind-generated waves. A similar phenomenon might have caused another major tsunami disaster in 1993 at the town of Hamatsumae in Okushiri Island, Japan: this town was also located in a sheltered zone of the island and was completely destroyed by 20-m-high tsunami run-up⁹. Significant tsunamis are rare phenomena behaving quite differently from everyday waves. Sufficient preparedness against such an event is formidably difficult to achieve, even though tsunami effects are predictable. □

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Selection against inbred song sparrows during a natural population bottleneck

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THE genetic and demographic consequences of population subdivision have received considerable attention from conservation biologists. In particular, losses of genetic variability and reduced viability and fecundity due to inbreeding (inbreeding depression) are of concern^{1–3}. Studies of domestic, laboratory^{4,5} and zoo populations^{2,6,7} have shown inbreeding depression in a variety of traits related to fitness. Consequently, inbreeding depression is widely accepted as a fact. Recently, however, the relative impact of inbreeding on the viability of natural populations has been questioned^{8–10}. Work on the cheetah (*Acinonyx jubatus*), for example, has emphasized the overwhelming importance of environmental factors on mortality in the wild^{9,10}. Here we report that song sparrows (*Melospiza melodia*) that survived a severe population bottleneck were a non-random subset of the pre-crash population with respect to inbreeding, and that natural selection favoured outbred individuals. Thus, inbreeding depression was expressed in the face of an environmental challenge. Such challenges are also likely to be faced by inbred populations of endangered species. We suggest that environmental and genetic effects on survival may interact and, as a consequence, that their effects on individuals and populations should not be considered independently.

Although inbreeding has been reported for several wild vertebrates¹¹, the few detailed studies of inbreeding done in the wild are equivocal^{12–16}. This could be explained by historical differences in the size and structure of populations. For example, purging of deleterious, recessive alleles from the genome is often put forward as a potentially positive effect resulting from periods of intense inbreeding following a severe bottleneck^{5,17}. Other work, however, suggests that even given severe bottlenecks, it may be difficult or impossible to eliminate detrimental mutations with individually small deleterious effects^{18–20}. We studied the resident population of song sparrows on Mandarte Island, British Columbia, from 1975 to 1990²¹. During this period the breeding density of the population fluctuated by 18-fold because of two major crashes in population size (Fig. 1). Both crashes occurred outside the breeding season, in 1979–1980 (9 females and 18 males surviving, corresponding to 18% of the adult population) and in 1988–1989 (4 females and 7 males surviving, corresponding to 11% of the adult population). Earlier accounts from Mandarte also report catastrophic mortality among adult song sparrows in 1962²². The 1962 and 1989 crashes were coincident with severe winter weather^{22,23}. The cause of the crash in 1980 is unknown. Immigration rates on Mandarte are low in ecological terms (16 immigrants in 16 years), but they are probably high enough to lead to near-panmixia in genetic terms⁵. There was no increased immigration after crashes²⁴.

Inbreeding coefficients were estimated on the basis of the pedigree (Fig. 1). Because their accuracy depends largely on the number of generations of known matings²⁵, for 1980, only five years after the initiation of our study, we underestimated the true inbreeding coefficients. Because of this, and a coincidental

TABLE 1 Mean inbreeding coefficient of adults and juveniles before and after the 1989 crash

	Adults	Juveniles
Before crash	0.0189 <i>n</i> = 74	0.0363 <i>n</i> = 132
After crash	0.0025 <i>n</i> = 7	0.0156 <i>n</i> = 3

n, Numbers of individuals with known grandparents in the sample.

gap in data collection (Fig. 1), we did not consider survival over the 1980 crash in detail.

During both the 1980 and 1989 crashes, the mean inbreeding coefficient in the breeding population was lower in the season after the crash than in the previous season (Fig. 1). This suggests that selection favoured birds that were relatively less inbred. To test this hypothesis, we compared the inbreeding coefficients of birds that survived the 1989 crash to those of birds that did not survive. As expected, we found that the average inbreeding coefficient was significantly lower among the birds that survived

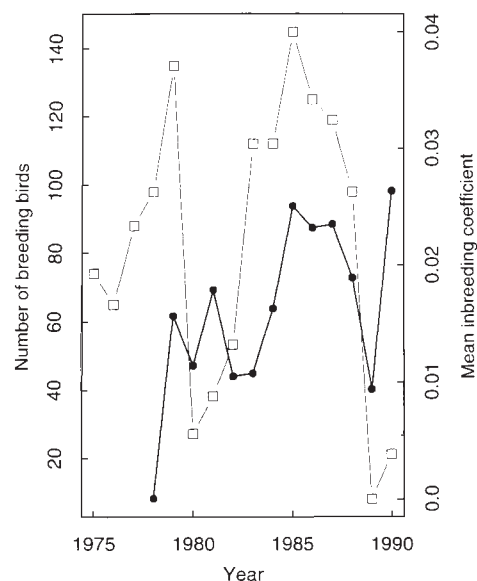


FIG. 1 The size of the song sparrow population breeding on Mandarte Island from 1975–1990 (squares) and the mean inbreeding coefficient of the population (circles). Because all individuals were colour-banded, survival was readily estimated²¹ and in 99.5% of 1,574 breeding attempts the putative parents were identified by detailed observations of pairing, mating and parental behaviour. Extra-pair paternity may have occurred, but several lines of evidence suggest it was rare²⁸. Continuous records of breeding success and adult and juvenile survival (except in 1980) allowed construction of a pedigree and the calculation of inbreeding coefficients. These were estimated using PEDSYS, a Pedigree Data Management System, provided by the Southwest Foundation for Biomedical Research, San Antonio, Texas, using the algorithm described in²⁹. We only used individuals whose grandparents were known to accommodate different depths of the pedigree due to founders and immigrants. This gave the following sample sizes for the calculations of mean *f* from 1978–1990: 9, 48, 11, 7, 12, 41, 52, 91, 93, 92, 74, 7 and 15. Incomplete data for 1980 led to a gap in the pedigree and resulted in low sample sizes of birds with known grandparents in 1981 and 1982. This contributed to lower mean inbreeding coefficients in these years. For 1975–1977 there were no breeding birds where all grandparents were known. Inbreeding coefficients are given relative to the founder population in 1975, assuming that all adults in 1975 and all subsequent immigrants had inbreeding coefficients of zero. The effects of this assumption on our analysis of the 1989 crash are small, because of the 11 intervening generations since the start of the study and the low level of immigration.

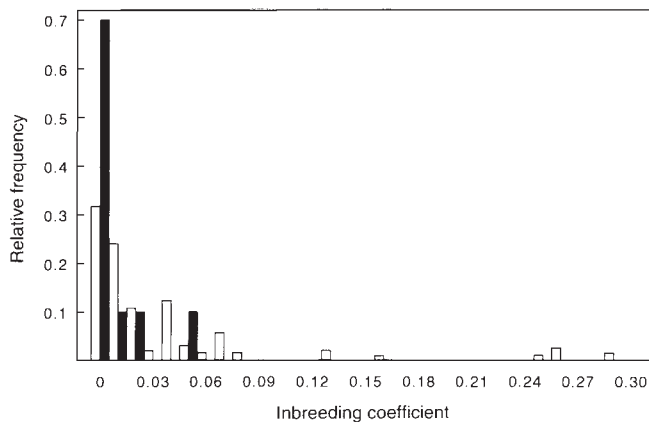


FIG. 2 The distribution of inbreeding coefficients of the birds that did not survive the crash (white bars) and of the survivors (black bars). The mean f of the birds that died equalled 0.0312 (s.d. = 0.059, $n = 196$), that of the survivors equalled 0.0065 (s.d. = 0.015, $n = 10$). The difference in the means of the two distributions was 0.0247 (two-tailed Mann-Whitney U -test, $Z = -2.24$, $P = 0.025$). The comparison was based on all adults and nestlings banded in 1988. Twelve birds survived the crash, but one was an immigrant and another had unknown maternal grandparents. Only eight of the survivors bred in 1989. Adult and juvenile birds were combined because of the small sample of survivors.

the crash than among birds that did not survive (Fig. 2). Only three of the survivors were inbred at all, two of which had low inbreeding coefficients ($f = 0.002$ and $f = 0.016$).

In a closed population, juveniles are expected to be more inbred on average than their parents. Hence differential mortality between juveniles and adults might be a confounding factor in our analysis, because juveniles typically survive less well than adults in our study population²¹. However, we found that the mean inbreeding coefficient was lower after the crash both among adults and juveniles (Table 1). This suggests that selection acted both against adults and juveniles, but the small number of survivors precluded a powerful test of this hypothesis. Nevertheless, our findings indicate that inbreeding depression occurred during a period of severe environmental stress. As a consequence of selection against inbred individuals, the mean inbreeding coefficient in the population was lower in the year following the bottleneck.

Our results show that inbreeding can affect survival in the wild. Given that the Mandarte population has undergone at least three population bottlenecks in the last three decades, our results also suggest that serial bottlenecks did not purge the genetic load sufficiently to alleviate the negative effects of inbreeding. Alternatively, immigration may be sufficient to re-establish deleterious alleles purged from the population during bottlenecks⁵. Finally, although genetic considerations should not be overemphasized^{9,26}, our results support the idea that genetic effects should not be considered independently of environmental effects²⁶. Our study shows that even in circumstances where most mortality can be attributed to environmental causes, genetic factors may still affect which individuals survive. In fact, inbreeding depression may be most pronounced exactly when individuals are environmentally stressed²⁷. This emphasizes the need to integrate demography and population genetics in studies in natural environments. □

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Non-spatial extinction following lesions of the parietal lobe in humans

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EFFICIENT behaviour in the visual environment requires selection between stimuli competing for control of action. Many current models of selection are spatial: relevant objects are chosen by attending to their locations^{1–3}. The unilateral stimulus extinction observed following lesions of the parietal lobe provides evidence for spatial selection⁴. Such patients may identify a single stimulus presented in their contralesional field, but can fail to detect the same stimulus when a competing stimulus is shown simultaneously on the ipsilesional side⁵. Here we demonstrate that extinction need not be spatial in nature, but may be determined by characteristics of the objects to be selected. In two patients with parietal lobe lesions and poor spatial localization, pictures extinguished words and closed shapes extinguished open shapes. This object-based extinction indicates the existence of biases within non-spatial selection mechanisms which are independent of biases produced by spatial selection mechanisms. We suggest that selection of objects for action requires that the 'winners' produced by the independent competitive biases for selection are bound together within distinct neural areas concerned with object properties and space.

We tested two patients: G.K., who suffered two strokes which resulted in lesions of the right occipito-parietal region and the left temporo-parietal region, and J.F., who suffered anoxia lead-

ing to lesion of the left parietal lobe above the lateral ventricle. Perimetric tests revealed that G.K. had full visual fields and J.F. a right homonymous hemianopia. Both patients had symptoms characteristic of Balint's syndrome, particularly in misreaching under visual guidance and in the inability to interpret complex pictures containing several objects^{6,7} (Fig. 1). Previous studies have demonstrated extinction in Balint's syndrome⁷, however, this could generally be due to attention becoming 'glued' to the location of the object first selected, following damage to a spatial system in which selection is based on object location⁸. Other studies have shown that lesions to the left and right cerebral hemispheres can generate problems in disengaging attention from objects and from space, respectively⁹, and that discrimination of visual elements can be impaired on one side of objects rather than of space¹⁰, but in each case the problems can still be attributed to impairments within a spatial selection system, albeit one that is influenced by the properties of objects⁸. Studies have not shown systematic biases in selection related to object properties when spatial selection is impaired and the localization of selected shapes is at chance, consistent with the idea of independent object-based and spatial biases in selection.

We first looked at stimulus extinction in G.K. and J.F. by presenting stimuli (either two words or two pictures) simultaneously above and below the fixation point. Though their ability to detect two stimuli was impaired compared with detection of

one (there was extinction), their reports indicated no spatial biases. However, when pictures were paired with words there was an obvious asymmetry in their performance; pictures extinguished words. This was unrelated to stimulus location. The patients were shown either single words, single pictures (line drawings of common objects or of non-identifiable shapes¹¹) or a picture with a spatially overlapping word (the double condition; see Fig. 1). The task was to report whether a word, a picture or both were present. The patients reported correctly in all single stimulus trials (40 trials with words, 20 trials each with objects and with nonsense shapes). G.K. reported both a picture and a word in 16/40 trials in the double condition, and just the picture in the remaining trials (9/20 with pictures of objects and 15/20 with nonsense shapes). He never detected just the word.

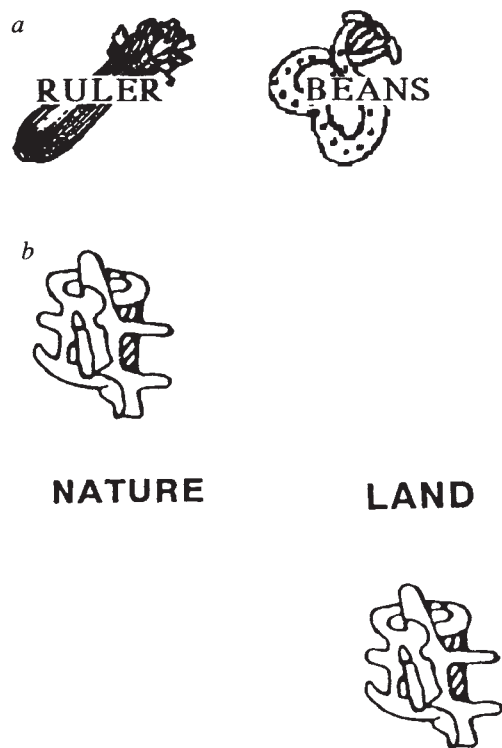


FIG. 1 Examples of the stimuli used in experiments 1 and 2. *a*, In experiment 1, words and pictures overlapped spatially (the double condition). *b*, In experiment 2 they did not overlap. In both cases, line drawings subtended an average visual angle of 3° high by 2.5° wide and words subtended an average visual angle of 2.5° by 0.5° . Stimuli were presented on a Macintosh LC microcomputer. At the start of each trial the patients were asked to fixate a central cross and fixation was checked visually. For both patients the display was presented unmasked for 2 s, centred at the point of fixation. In experiment 1 there were 40 single words, to match the total number of single pictures (20 of real objects, 20 of nonsense shapes). There were 20 double trials with pictures of real objects and 20 with nonsense shapes. In experiment 2 words alone were centred at the fixation point and pictures were centred 3° above or below fixation. The exposure duration was the same as for experiment 1. Presentations above and below fixation were used to minimize effects of any lateral field defect or preferences.

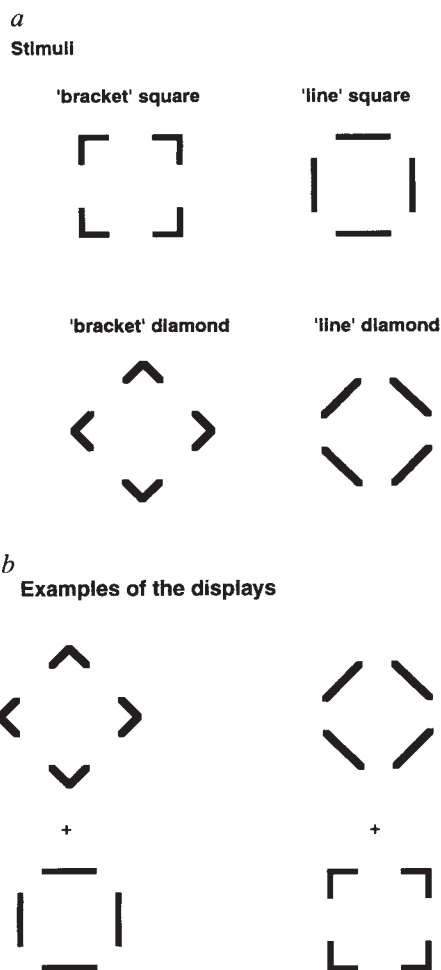


FIG. 2 *a*, Examples of the stimuli used in experiment 3: 'bracket-' and 'line-' squares and diamonds. Each shape subtended a visual angle of 1° , and was centred either 2° above or below the fixation point. G.K. and J.F. each fixated a central cross at the start of each trial and fixation was checked visually. The fixation cross was offset when the square and/or diamond shapes were presented. For G.K. the exposure duration of the square and diamond shapes was 480 ms; for J.F. it was 1.5 s. For normal subjects, both detection and localization of the square is trivially easy under these exposure conditions. There were 12 trials with single squares (6 with bracket-squares and 6 with line-squares) and 12 with single diamonds (6 bracket and 6 line shapes), and 24 trials with double shapes, in which either a bracket-square was paired with a line-diamond (12 trials) or a line-square was paired with a bracket-diamond (12 trials). *b*, Examples of displays used in the double shapes condition. With such displays G.K. and J.F. both failed to detect the line-square (paired with the bracket-diamond) though they detected the bracket-square (paired with the line-diamond); this occurred irrespective of whether squares appeared above or (as shown here) below fixation.

J.F. detected both stimuli on 12/40 trials (5 with real objects, 7 with nonsense shapes), just the picture on 23 trials (12 with real objects, 11 with nonsense shapes) and just the word on 5 trials (3 with real objects, 2 with nonsense shapes). Although performance was good with single items, a word presented concurrently with a picture was likely to be extinguished.

The second experiment assessed G.K.'s performance when selection biases based on the nature of the stimulus (whether it was a picture or a word) were pitted against a spatial selection bias (favouring the fixated stimulus)¹². Words were shown at the point of fixation and pictures (when present) shown simultaneously, either above or below (Fig. 1). G.K. was asked to read the fixated word and to ignore the picture. He read successfully 39 of 45 single words, 15 of 45 words presented with pictures of objects and 12 of the 45 with nonsense shapes. He failed to report the presence of any word in 22 trials with the pictures of objects and 20 with nonsense shapes. Thus, G.K. again showed extinction of words presented simultaneously with pictures, though spatial selection would favour the words.

Pictures might dominate visual selection over words because their meaning is retrieved more quickly¹³; pictures are darker and more complex and they are closed figures. But as equivalent effects were found with pictures of real objects and nonsense shapes, speed of retrieving meaning was probably not crucial. Our third experiment assessed the effect of shape 'closure' by comparing shapes matched for size and amount of contour but that differed in their degree of closure. Displays contained a single square, a single diamond, or a square and a diamond. Single shapes appeared randomly above or below the fixation point, and on 'double shape' trials one was above and the other below the fixation point (in half of the trials the square was above and in half below). The task was to detect whether a square was present. The shapes were constructed from the corner 'brackets' or from the 'lines' comprising the edges of the forms (Fig. 2). Closure can be computed more easily between than across corner junctions¹⁴; accordingly, there should be an advantage for computing closure with 'brackets' relative to 'lines'. In half of the double-shape trials, bracket-squares were paired with line-diamonds, and in the other half, line-squares were paired with bracket-diamonds. Following the detection of a square, both patients had to decide whether the square appeared above or below the fixation point, to allow assessment of the spatial localization of selected shapes.

Subjects G.K. and J.F. both showed a marked preference for selecting bracket-shapes (where closure was easy to compute) over line-shapes (where closure was relatively difficult to compute) when bracket- and line-shapes were pitted against one another (Fig. 3). Location decisions about selected squares were

at chance. The presence of closure in pictures, but not in words may account for the selective extinction of words by pictures.

As in previous cases of Balint's syndrome, both patients showed stimulus extinction. The experiments show that extinction can be based on object properties such as closure, and that selection based on these properties occurred even when spatial selection and localization were poor. G.K. and J.F. suffered lesions of the parietal lobes, which may normally mediate spatial selection in vision. In contrast, occipito-temporal (ventral) visual areas, mediating the processing of object properties¹⁵, were relatively intact. Closed shapes may dominate in competition for selection over open shapes within ventral areas specialized for object processing. This object-based competition may be revealed under conditions such as those explored here for the two patients, when the lesioned spatial selection system does not modulate performance. Extinction is demonstrated because the lesioned spatial selection system may be important for switching attention between objects. Though the object and the spatial selection systems can be functionally separated, we suggest that there is normally coordination of the outcomes of competition within the separate neural areas coding each property, making the shape, location and other properties of a single object available concurrently for the control of behaviour¹⁶. □

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Specific incorporation of cyclophilin A into HIV-1 virions

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LITTLE is known about host factors necessary for retroviral virion assembly or uncoating. We have previously shown that the principal structural protein of the human immunodeficiency virus HIV-1, the Gag polyprotein, binds the cyclophilin peptidyl-prolyl isomerases¹; cyclophilins catalyse a rate-limiting step in protein folding² and protect cells from heat shock³. Here we demonstrate that cyclophilin A is specifically incorporated into HIV-1 virions but not into virions of other primate immunodeficiency viruses. A proline-rich region conserved in all HIV-1 Gag polyproteins is required for cyclophilin A binding and incorporation. Disruption of a single proline blocks the Gag-cyclophilin interaction *in vitro*, prevents cyclophilin A incorporation into virions, and inhibits HIV-1 replication. Our results indicate that the interaction of Gag with cyclophilin A is necessary for the formation of infectious HIV-1 virions.

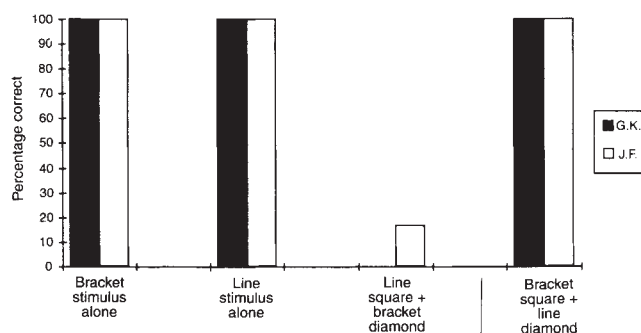


FIG. 3 The percentage of correct detection responses to squares made by G.K. and J.F. There was good detection of single bracket- and single line-squares, and good detection of bracket-squares paired with line-diamonds, but poor detection of line-squares paired with bracket-diamonds. On trials with correct detections of squares, G.K. made 13/24 correct location decisions (above or below fixation); J.F. made 14/26 correct location decisions. These location decisions were at a chance level.

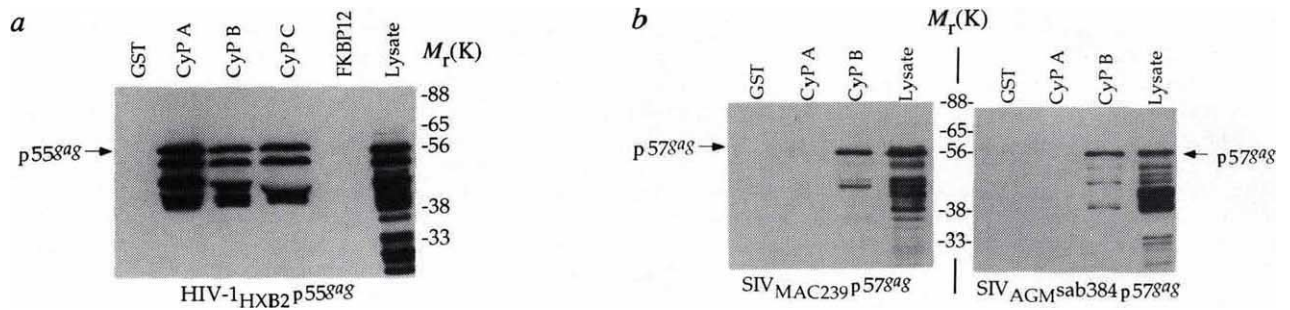


FIG. 1 Binding of primate immunodeficiency virus Gag polyproteins to immunophilins *in vitro*. Lysate from bacteria expressing a, the Gag polyprotein of HIV-1_{HXB2}, or b, the Gag polyproteins of SIV_{MAC239} and SIV_{AGMsab384}, was mixed with lysate from bacteria expressing glutathione S-transferase (GST) or GST fused to the indicated immunophilins. GST proteins were collected on glutathione-agarose beads. Bound protein was analysed by SDS-PAGE and visualized by western blotting using anti-Gag antibody. Total lysate from bacteria expressing the indicated Gag polyprotein is shown. Migration positions of molecular size markers are indicated.

METHODS. The HIV-1_{HXB2} p55^{gag} expression construct (pT7HG-Pro-) has been described¹⁰. To express SIV_{MAC239} p57^{gag} in bacteria, the HIV-1

gag gene from pT7HG-Pro- was replaced with the SIV_{MAC239} *gag* gene using previously engineered *Nde*I and *Sal*I sites¹¹. To express SIV_{AGMsab} p57^{gag} (ref. 12), the *gag* gene was cloned into the plasmid pSE420 (Invitrogen) using previously engineered *Nco*I and *Eco*RI sites (from B. Hahn). The GST-cyclophilin A and B expression constructs have been described¹; GST-cyclophilin C¹³ was from M. Trahey and I. Weissman; GST-FKBP12 (ref. 14) was from R. Bram and G. Crabtree. Protein induction, bacterial lysis, binding reactions, gel electrophoresis, and immunoblotting were all done as before¹. The primary antibody used in westerns was a monoclonal anti-HIV-2 Gag antibody which reacts equally well with HIV-1 Gag (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH; donated by P. Hoshikawa).

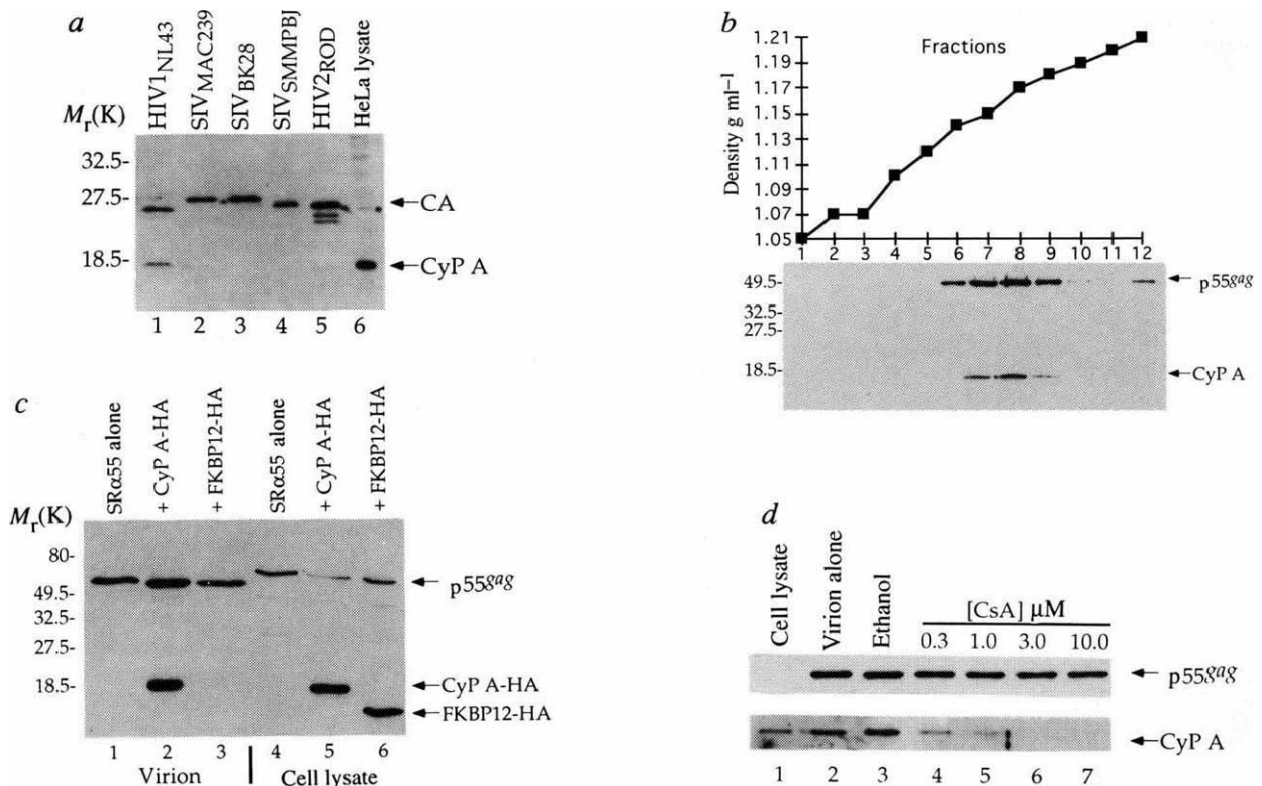


FIG. 2 Specific incorporation of cyclophilin A into HIV-1 virions. Immunoblots of virion protein from a, the supernatant of HeLa cells transfected with the indicated proviral DNAs; b, sucrose density gradient fractions of supernatant from COS cells transfected with the Gag polyprotein expression construct pSRα55 and a haemagglutinin-tagged cyclophilin A expression construct; c, lanes 1-3, supernatant of COS cells transfected with the indicated plasmids (cell lysate in lanes 4-6); and d, supernatant from COS cells transfected with pSRα55 in the presence of cyclosporin A. Primary antibodies used in western blots were: a, d, anti-Gag and anti-cyclophilin A; b, c, anti-Gag and anti-HA. The migration positions of p55^{gag}, capsid (CA), cyclophilin A (CyP A), FKBP12 and of molecular size markers is shown.

METHODS. The infectious proviral constructs have been described^{12,15-19}. pSRα55 uses the SRα promoter of pBEX1 (ref. 20)

to express Rev-independent *gag* sequences (from G. Pavlakakis and B. Felber, manuscript in preparation), and has been partially characterized²¹. The haemagglutinin-tagged cyclophilin A and FKBP12 expression plasmids²⁰ were from R. Bram. DNA was transfected into HeLa and COS cells (ATCC) by calcium phosphate precipitation²². Virions were purified through 25% sucrose²³ and density gradients were analysed²⁴ as described. Cells were lysed in 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.5). Cyclosporin A (Sandoz) was dissolved in ethanol and added to culture medium at the indicated concentrations. Rabbit anti-cyclophilin A antibody was raised against purified GST-cyclophilin A expressed in bacteria¹. Monoclonal antibody 12CA5 (Babco) recognizes the influenza virus haemagglutinin epitope²⁵. The anti-Gag antibody is described in Fig. 1 legend.

HIV-1 Gag polyprotein binds *in vitro* to cyclophilins A, B and C, but not to the unrelated prolyl isomerase FKBP12 (Fig. 1a). In contrast, the Gag polyproteins encoded by two species of simian immunodeficiency virus, SIV_{MAC239} and SIV_{AGMab384}, bind to cyclophilin B but not to cyclophilin A (Fig. 1b). Unlike the cytoplasmic protein cyclophilin A, cyclophilin B is targeted to the endoplasmic reticulum⁴, and probably does not play any part in Gag function. These results suggest that among primate immunodeficiency viruses, only HIV-1 replication might require the cyclophilin interaction.

Purified HIV-1 virions contain protein of relative molecular mass 18,000 (18K) which reacts with an anti-cyclophilin A antibody (Fig. 2a, lane 1). Radiolabelling of virion protein indicates that there are ten Gag molecules for each cyclophilin A in the virion (data not shown), which represents at least as much virion-associated cyclophilin A as *pol*-encoded proteins⁵. Virions produced by three cloned SIV proviruses and one HIV-2 provirus do not incorporate detectable amounts of cyclophilin A (Fig. 2a, lanes 2–5) irrespective of whether virions are produced in human or simian cell lines. Thus, incorporation of cyclophilin into virions correlates with the ability of the Gag polyprotein to bind to cyclophilin A *in vitro*.

Cyclophilin A copurifies on a linear sucrose gradient with virions produced by an expression vector in which the *gag* open reading frame is the only HIV-1 genetic element (Fig. 2b). This indicates that HIV-1 Gag polyprotein is sufficient for packaging of cyclophilin A into virions. To exclude the possibility that the HIV-1 Gag polyprotein nonspecifically binds cytoplasmic proteins, we compared the virion incorporation of different proteins expressed as fusions with the same epitope tag. Cyclophilin A fused to the epitope tag was easily detected in virions (Fig. 2c, lane 2), but the cytoplasmic prolyl isomerase FKBP12 could not be detected (Fig. 2c, lane 3). Thus, cyclophilin A is not incorporated into HIV-1 virions simply because it is an abundant cytoplasmic protein. Also, cyclosporin A, which blocks the Gag–cyclophilin interaction *in vitro*¹, prevents cyclophilin A incorporation into virions (Fig. 2d). These experiments demonstrate that cyclophilin A is specifically incorporated into HIV-1 virions through contacts with the Gag polyprotein.

Amino acids in the first half of the HIV-1 capsid are necessary for binding cyclophilin A and B¹. To determine which HIV-1 sequences are specifically required for binding to cyclophilin A, HIV-1/SIV *gag* chimaeras were engineered

in which coding sequences from this domain were exchanged (Fig. 3a). An 88-nucleotide fragment from HIV-1 was transferred into the corresponding position of SIV *gag*: the encoded protein could bind cyclophilin A (Fig. 3b, lane 6). The analogous fragment from SIV disrupted the ability of HIV-1 Gag to bind cyclophilin A (Fig. 3b, lane 2). All six chimaeras gave results consistent with the conclusion that the 88-nucleotide region from HIV-1 encodes information necessary for binding cyclophilin A (Fig. 3a).

Examination of the amino-acid sequence encoded by the 88-nucleotide fragment of HIV-1 revealed an array of prolines (PX₄PX₂PX₅P) that is conserved in all HIV-1 isolates⁶. Critical differences were evident when this region was aligned with sequences from other primate immunodeficiency viruses (Fig. 4a), suggesting that the proline array might be necessary for HIV-1 Gag binding to the prolyl-isomerase cyclophilin A. We therefore tested four mutant HIV-1 Gag polyproteins, each with a different proline changed to an alanine, for their ability to bind cyclophilin A. Three of the mutants, P217A, P225A and P231A, had no effect on binding (Fig. 4b; lanes 6, 14 and 18). Mutant P222A disrupted binding to cyclophilin A, but did not affect binding to cyclophilin B (Fig. 4b; lanes 10 and 11). Thus, P222A converted HIV-1 Gag to an SIV phenotype. Although SIV isolates have a proline at the equivalent position, our data suggest that P222, as found within the context of the HIV-1 proline array, is necessary for HIV-1 Gag polyprotein binding to cyclophilin A.

We tested the importance of the 88-nucleotide fragment and of each of the proline mutants for the incorporation of cyclophilin A into virions. When the 88-nucleotide fragment from HIV-1 was replaced with the corresponding fragment from SIV, cyclophilin A incorporation was disrupted (Fig. 4c, lane 3). Similarly, virions produced by mutant P222A could not incorporate cyclophilin A (Fig. 4c, lane 5). The other three proline mutants behaved like wild type (Fig. 4c; lanes 4, 6 and 7). We conclude that the same Gag sequences required for binding to cyclophilin A *in vitro* are necessary for packaging cyclophilin A into virions.

Finally, we examined the importance of each of the four prolines for HIV-1 replication (Fig. 4d). Mutants P217A and P231A did not replicate in Jurkat cells, but these mutants decreased particle yield four- and tenfold, respectively. Mutant P225A replicated with wild-type kinetics. P222A, the mutant with normal particle yield but which could not incorporate cyclophilin A,

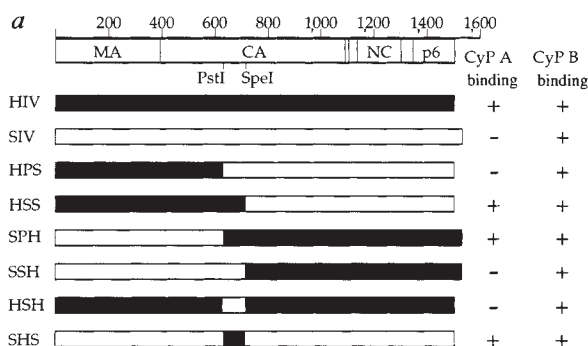
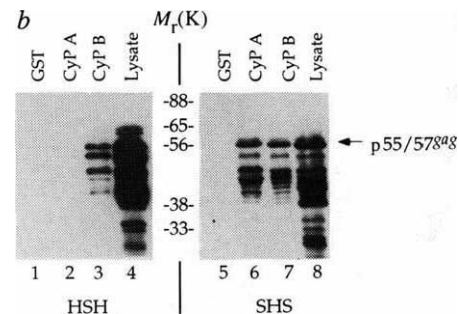


FIG. 3 An 88-nucleotide HIV-1 *gag* coding fragment is necessary for Gag polyprotein binding to cyclophilin A. **a**, Diagram showing the ability of various HIV-1/SIV *gag* chimaeras to bind to cyclophilin A and B *in vitro*. **b**, Representative binding experiment showing two of the HIV-1/SIV chimaeric Gag polyproteins.

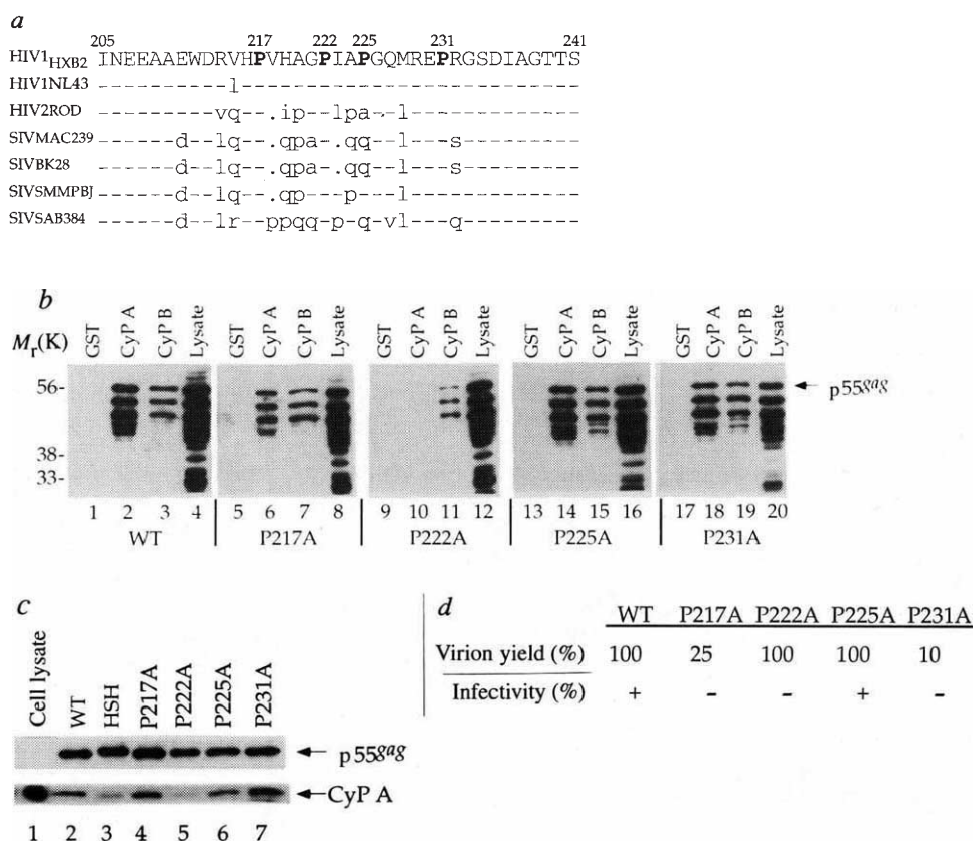
METHODS. HIV-1/SIV chimaeras were constructed by standard recombinant DNA techniques using conserved *Pst*I and *Spe*I sites in



HIV-1_{HXB2} and SIV_{MAC239} (HIV-1_{HXB2} nucleotide positions 1,411–1,509). The diagram shows sequences contained by different chimaeras. Black bars indicate HIV-1 sequences; white bars indicate SIV sequences. Expression of the HIV-1/SIV Gag polyprotein chimaeric proteins, binding reactions, gel electrophoresis and immunoblotting are described in Fig. 1 legend.

FIG. 4 HIV-1 Gag polyprotein P222 is required for cyclophilin A binding, cyclophilin A incorporation into virions, and for viral replication. *a*, Amino-acid sequence alignment between the Gag polyproteins of HIV-1 and other primate immunodeficiency viruses. The proline-rich region required for cyclophilin A binding and virion incorporation is shown. Numbering is with respect to the amino terminus of the HIV-1_{HXB2} Gag polyprotein. Conserved prolines are highlighted. Absolutely conserved positions are indicated by dashes, sequence gaps by dots. *b*, Immunoblot showing binding to GST-cyclophilins of bacterially expressed HIV-1_{HXB2} Gag polyprotein containing the indicated mutations. *c*, Immunoblot showing the incorporation of native cyclophilin A into virion particles produced by pSR α 55 containing the indicated mutations. *d*, Phenotype of virus containing each of the four proline mutations. Virion yield is relative to wild type. +, Wild-type peak reverse transcriptase activity in cell culture supernatant by day 5; -, no significant reverse transcriptase activity by day 14. WT, wild type.

METHODS. *In vitro* binding assays and assays for the incorporation of cyclophilin A into virions are described in legends to Figs 1 and 2. The mutants P217A, P222A, P225A and P231A were constructed with the following oligonucleotides using the Transformer site-directed mutagenesis kit (Clontech): 5'-CCCTGCATGTACAGCATGCACTCTATCCCA-3', 5'-CCTGGTCAATTGCCCTGCATGC-3', 5'-CTCATCTGGCCGGCTGCAATAGGC-3'



did not replicate, consistent with a requirement for cyclophilin A incorporation into virions for HIV-1 replication.

These experiments demonstrate that cyclophilin A is specifically incorporated into HIV-1 virions through contacts with the Gag polyprotein, and suggest that the Gag-cyclophilin A interaction is necessary for HIV-1 replication. Mutation of a single Gag proline disrupts the Gag-cyclophilin interaction *in vitro*, cyclophilin A incorporation into virions, and viral replication. Cyclosporin A also blocks the Gag-cyclophilin interaction *in vitro*¹, cyclophilin A incorporation into virions, and the replication of HIV-1. These effects require drug concentrations that are 10–100-fold higher than are necessary for immunosuppression but which are the same as the concentration of cyclophilin A in

and 5'-GCTATGTCACCTCCACGTGCTCTCTCATCTG-3'. The effect of each mutant on the replication of the HIV-1 clone NL4-3 in the Jurkat T-cell line was determined as described²⁶.

the cytoplasm⁷. The same effects on Gag-cyclophilin binding and HIV-1 replication were observed with comparable doses of two non-immunosuppressive cyclosporin A analogues, but not with the structurally unrelated immunosuppressant FK506 (ref. 8; M. Thali, manuscript in preparation, and E.K.F. and J.L., unpublished results). Cyclosporin A does not inhibit the replication of related retroviruses such as SIV (ref. 9; M. Thali, in preparation, and E.K.F. and J.L., unpublished), which do not encode Gag polyproteins capable of binding cyclophilin A. Our data suggest that cyclosporin A blocks HIV-1 replication by competing directly with Gag for binding to cyclophilin A, and that HIV-1 virion infectivity uniquely requires the formation of a Gag-cyclophilin A complex. □

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Functional association of cyclophilin A with HIV-1 virions

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CYCLOPHILINS are a family of proteins that bind the immunosuppressant cyclosporin A, possess peptidyl-prolyl *cis-trans* isomerase activity, and assist in the folding of proteins¹⁻⁶. Human cyclophilins A and B are host cell proteins that bind specifically to the HIV-1 Gag polyprotein p55^{gag} *in vitro*⁷. Here we report that viral particles formed by p55^{gag}, in contrast to particles formed by the Gag polyproteins of other retroviruses, contain significant amounts of cyclophilin A. Sequences in the capsid domain of p55^{gag} are both required and sufficient for the virion-association of cyclophilin A. The association of cyclophilin A with HIV-1 virions was inhibited in a dose-dependent manner by cyclosporin A as well as by SDZ NIM811 ([Melle-4]cyclosporin), a non-immunosuppressive analogue of cyclosporin A⁸. Drug-induced reductions in virion-associated cyclophilin A levels were accompanied by reductions in virion infectivity, indicating that the association is functionally relevant. Moreover, SDZ NIM811 inhibited the replication of HIV-1 but was inactive against SIV_{MAC}, a primate immunodeficiency virus closely related to HIV-1, which does not incorporate cyclophilin A.

To identify cellular proteins associated with HIV-1 virions, HeLa cells transfected with HIV-1 proviral DNA were metabolically labelled with [³⁵S]methionine and viral particles released into the supernatant were pelleted through sucrose. Analysis of the virion lysate by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of significant amounts of a virion-associated protein of relative molecular mass 18K, in addition to viral structural proteins (Fig. 1a, upper panels, lanes 2 and 5). The 18K band did not represent a Gag cleavage product, because the transfected provirus carried a point mutation in the protease gene to prevent proteolytic processing of the Gag polyprotein p55^{gag} and the Gag-Pol polyprotein p160^{gag-pol} (ref. 9). The recent finding that p55^{gag} binds tightly to cyclophilins *in vitro*⁷ led us to investigate whether the virion-associated 18K protein represented a cyclophilin. As shown in Fig. 1a (lower panels), the 18K protein could be immunoprecipitated from HIV-1 virions by an antiserum directed against human cyclophilin A, but not by control sera (not shown). The 18K protein in HIV-1 virion preparations co-sedimented with viral particles in sucrose gradients, confirming its association with virions, and co-migrated exactly with cyclophilin A immunoprecipitated from HeLa cell lysates in SDS-PAGE gels (not shown). A comparison of the band intensities in Fig. 1a by densitometry, taking into account the number of methionine residues in each molecule, suggested that the molar ratio of cyclophilin A to p55^{gag} in the HIV-1 virion lysates was ~1:10, a ratio similar to that of p160^{gag-pol} to p55^{gag}. To determine whether cyclophilin A is associated with other retrovirus particles, HeLa cells were transfected with plasmids expressing the Gag polyproteins of SIV_{MAC}, HIV-2, visna virus, and murine Moloney leukaemia virus (Mo-MuLV). In each case, viral particles were released

that contained Gag polyproteins of the expected electrophoretic mobility, but virtually no cyclophilin A (Fig. 1a). This result is consistent with the finding in the yeast GAL4 two-hybrid system that only the Gag polyprotein of HIV-1, but not that of SIV_{MAC} or other retroviruses, interacts with human cyclophilin A (ref. 7).

The p55^{gag}-cyclophilin A interaction in the yeast two-hybrid system was disrupted by linker insertion mutations affecting the N-terminal half of the capsid domain of p55^{gag} (ref. 7). The capsid domain forms the cone-shaped core of the mature HIV-1 virion¹⁰. Mutations affecting the capsid domain of p55^{gag}, which prevent virus replication when introduced into a replication-competent molecular clone of HIV-1 (refs 11, 23), were examined for an effect on cyclophilin A association with HIV-1 virions (Fig. 1b). All of the mutant p55^{gag} expressor constructs retained the ability to produce viral particles with an efficiency similar to that of the parental construct upon transfection into HeLa cells, as judged from the amount of p55^{gag} detectable in pelleted virion preparations (Fig. 1b, upper panel). A substitution in the major homology region (MHR) of the capsid domain, a stretch of 20 amino acids uniquely conserved among retroviruses¹², had no effect on the amount of virion-associated cyclophilin A (Fig. 1b, lane 8). By contrast, the ratio of cyclophilin A to p55^{gag} in virion lysates was drastically reduced by each of five small in-frame deletions in the capsid domain upstream of the MHR (Fig. 1b, lanes 3-7). These results demonstrate that sequences within the N-terminal two thirds of the capsid domain are required for the virion-association of cyclophilin A. Sequences within the HIV-1 capsid domain are also sufficient to mediate the virion-association of cyclophilin A as the replacement of the capsid domain of the Mo-MuLV Gag polyprotein by the capsid domain of HIV-1 resulted in the formation of particles which, unlike particles formed by the parental MuLV Gag polyprotein, contained cyclophilin A at levels comparable to those found in HIV-1 virions (Fig. 1c).

The *in vitro* binding of p55^{gag} to cyclophilin A could be inhibited by cyclosporin A (CsA)⁷, a cyclic undecapeptide that binds to the prolyl isomerase substrate-binding site of cyclophilin A⁵. Figure 2 shows that although CsA has no effect on viral particle production in HeLa cells, the virion association of cyclophilin A is markedly inhibited. A dose-dependent reduction in the ratio of cyclophilin A to p55^{gag} in virions was apparent at CsA concentrations at or above 0.5 µM. The association of cyclophilin A with HIV-1 virions was similarly inhibited by SDZ NIM811 ([Melle-4]cyclosporin), a natural CsA analogue that is devoid of immunosuppressive activity but retains the capacity to bind to cyclophilin A⁸. Drug-induced reductions in virion-associated cyclophilin A levels were paralleled by reductions in virion infectivity (Fig. 3) as measured by transfer of chloramphenicol acetyltransferase (CAT) under conditions that permit only a single round of virus transmission¹³. When the target cells rather than the producer cells were incubated with CsA or SDZ NIM811 for 24 h before infection and up to 10 h after infection, minimal effects on the efficiency of CAT transfer that were not dose-dependent were seen (data not shown). These results indicate that the major specific inhibitory effect of these drugs on HIV-1 transmission involves interaction with the virus-producing cells. The effect of the cyclophilin-binding drugs on HIV-1 virion infectivity was independent of their immunosuppressive activity, because comparable results were obtained with CsA and SDZ NIM811 (Fig. 3). As illustrated in Fig. 4, the non-immunosuppressive CsA analogue significantly inhibited the spread of HIV-1 in an infected culture at concentrations as low as 0.5 µM, whereas the spread of SIV_{MAC} in the same culture system was largely unaffected even at 2.5 µM.

A selective association of substantial amounts of cellular surface antigens such as β₂-microglobulin and human lymphocyte antigen DR with HIV-1 and other primate immunodeficiency viruses has been reported¹⁴⁻¹⁷. By contrast, the propensity to incorporate stoichiometrically significant amounts of cyclophilin

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FIG. 1 Virion-association of cyclophilin A (CyPA). Viral particles produced in HeLa cells were analysed directly by SDS-PAGE (upper panels) and in parallel by immunoprecipitation with a rabbit anti-human cyclophilin A antiserum¹⁸ followed by SDS-PAGE (lower panels). a, Selective association of cyclophilin A with HIV-1 virions. Proteins associated with protease-defective HIV-1 virions and viral particles formed by the Gag polyproteins of SIV_{MAC239}, HIV-2_{ROD}, visna virus and Moloney MuLV are compared.

b, Requirement for the capsid (CA) domain of the HIV-1 Gag polyprotein p55^{gag} for the virion association of cyclophilin A. Comparison of proteins associated with viral particles formed by wild-type and mutant HIV-1 Gag polyproteins. Numbers above each lane refer to the position of deleted or altered amino acids in p55^{gag} relative to the N terminus of the CA domain. c, The presence of the HIV-1 CA domain in a heterologous Gag polyprotein is sufficient for the association of cyclophilin A with viral particles. Comparison of proteins associated with particles formed by the parental HIV-1 and Mo-MuLV Gag polyproteins and a chimaeric MuLV Gag polyprotein which has the CA domain replaced by the CA domain and p2-spacer peptide of HIV-1. d, Domain organization of the parental and chimaeric Gag polyproteins and location of mutations in the HIV-1 CA domain. MA, matrix domain; NC, nucleocapsid domain; MHR, major homology region.

METHODS. HeLa cells were transfected with Gag polyprotein expression plasmids, metabolically labelled with [³⁵S]methionine from 48 to 60 h post-transfection, and viral particles released during the labelling period were pelleted through 20% sucrose cushions and lysed as described¹⁹. To express the HIV-1 Gag polyprotein p55^{gag}, the protease-defective HXBH10-PR⁻ mutant of the replication-competent HXBH10 molecular clone of HIV-1 was used²⁰. To express the SIV_{MAC239} Gag polyprotein, a fragment containing the gag region of SIV_{MAC239} (nt 1,080–3,034) was inserted into HXBH10 between nt 637 and 5,228. To express the Gag polyproteins of HIV-2_{ROD}, visna virus, and Mo-MuLV, the viral protease gene in the chimaeric proviral constructs HXBH10/ROD^{gag-pol}, HXBH10/LV^{gag-pol} and HXBH10/NCA^{gag} (ref. 20) was disrupted by frameshift mutations. The construction of the R167→A substitution and of the panel of deletion mutations in the CA coding sequence of the HIV-1 gag gene has been described^{11,23}. The CA coding region of the Mo-MuLV gag gene (nt 1,266–2,054) in HXBH10/NCA^{gag} was replaced by the corresponding sequence from HIV-1_{HXB2} (nt 1,185–1,919) using blunt-end cloning sites at the domain boundaries, which were created by site-directed mutagenesis. The resulting chimaeric gag gene contains one translationally silent mutation (C to T) at position 1,265 of the MuLV-derived sequence. A minus sign indicates that the HXBH10-gag⁻ mutant was transfected, which is identical to HXBH10 except for premature termination codons in the gag gene¹⁹.

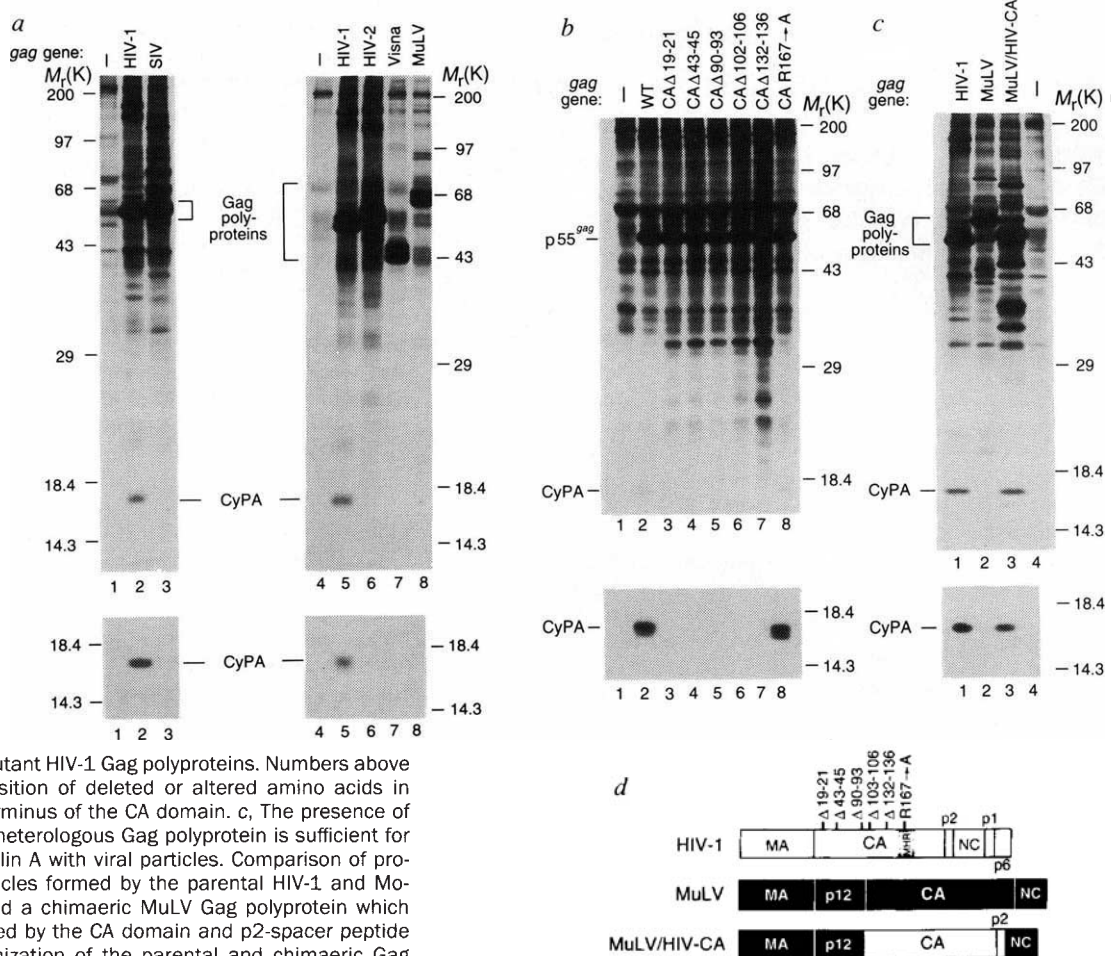
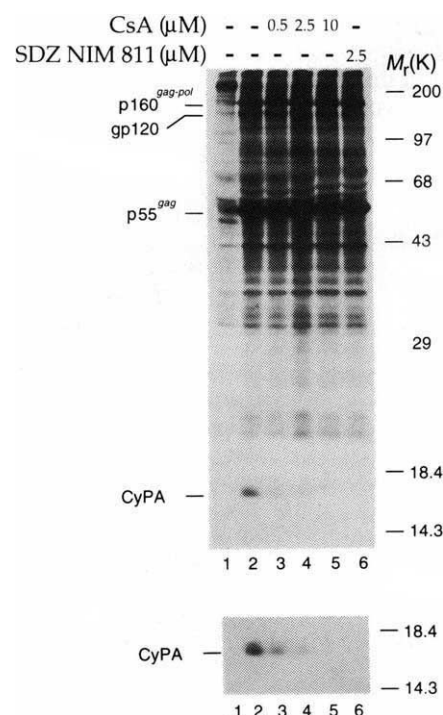


FIG. 2 Effect of cyclophilin-binding drugs on the virion-association of cyclophilin A. Proteins associated with HIV-1 virions produced in the presence of the indicated concentrations of CsA or SDZ NIM811 were analysed directly by SDS-PAGE (upper panel) and by immunoprecipitation with anti-cyclophilin A antiserum (lower panel).

METHODS. HeLa cells were transfected with HXBH10-gag⁻ (lane 1) or the protease-defective mutant HXBH10-PR⁻ (lanes 2–6). Transfected cells were metabolically labelled and viral particles prepared as described in Fig. 1 legend, except that CsA or SDZ NIM811 (Sandoz) dissolved in dimethylsulphoxide (DMSO), or DMSO alone, were present during the labelling period.



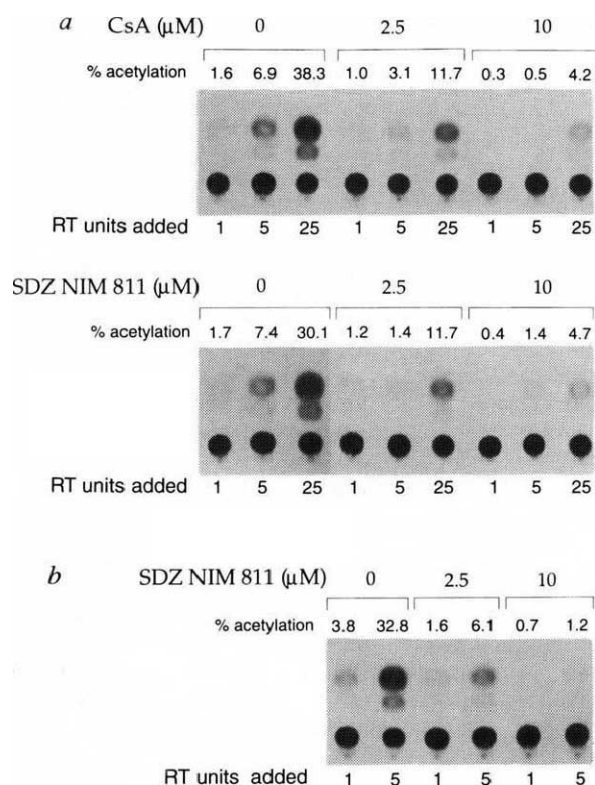
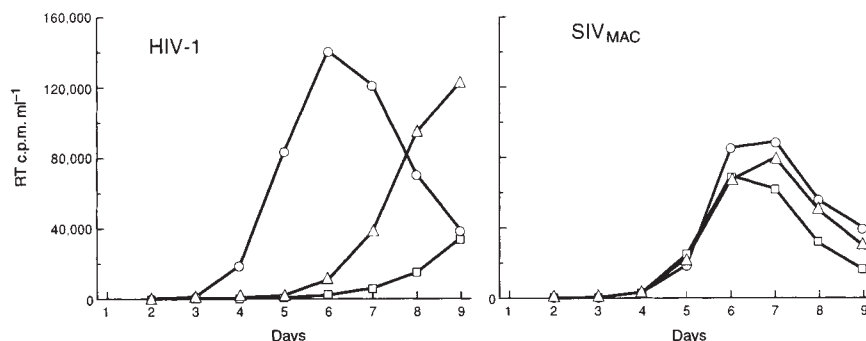


FIG. 3 Effect of cyclophilin-binding drugs on HIV-1 virion infectivity. Infection by an HIV-1 virus that expresses a bacterial chloramphenicol acetyltransferase (CAT) enzyme was monitored by measuring CAT activity in target cells. To prevent further transmission after a single round of virus replication, a defective HIV-1 genome dependent on *trans*-complementation by envelope glycoprotein was used. *a*, CAT activity following transfer of COS-1 cell-produced virions onto Jurkat lymphocytes. *b*, CAT activity following transfer of HeLa cell-produced virions onto phytohaemagglutinin-stimulated human peripheral blood mononuclear cells.

METHODS. COS-1 or HeLa cells were cotransfected with an HIV-1 envelope glycoprotein expression plasmid (pSVIIenvMN) and a plasmid containing an HIV-1 provirus with a deletion in the *env* gene and a CAT gene replacing the *nef* gene (pXHB10 Δ envCAT) as described¹³. At 48 h post-transfection, the culture medium was replaced by medium containing CsA or SDZ NIM811 dissolved in DMSO, or DMSO alone. At 60 h post-transfection, supernatants were collected from the producer cells, adjusted to a final concentration of 10 μ M CsA or SDZ NIM811, and virions released during the 12 h incubation period were purified through 20% sucrose. Equivalent amounts of reverse transcriptase units (1, 5 or 25 $\times 10^3$ c.p.m.) were added to CD4⁺ cells and CAT activity in the target cells determined as described²¹. The pSVIIenvMN expression plasmid (gift from J. Li) was derived from pSVIIenv (ref. 13) by replacing nt, 6,346–8,059 of the HIV-1_{HXB2} *env* gene with the corresponding sequence from the prototypic HIV-1_{MN} strain (from P. Berman, Genentech). The pXHB10 Δ envCAT proviral construct was obtained by replacing nt 5,372–5,934 of the HIV-1_{HXB2}-derived sequence in pXHB Δ envCAT (ref. 13) with the corresponding sequence from HIV-1_{BH10}, thereby introducing a functional *vpu* gene.

FIG. 4 Comparison of the effect of the non-immunosuppressive CsA analogue SDZ NIM811 on HIV-1 and SIV_{MAC} replication. Fresh CEMX174 cells (4×10^6) were exposed for 2 h to supernatant from CEMX174 cells chronically infected with HIV-1_{HXB2} or SIV_{MAC239} containing 2×10^4 c.p.m. reverse transcriptase activity. Cultures were then split and maintained in the absence of drug ($\circ$) or in the presence of either 0.5 μ M (Δ) or 2.5 μ M ($\square$) SDZ NIM811. Viral replication was monitored daily by measuring particle-associated reverse transcriptase activity in culture supernatants as described²².



A distinguishes HIV-1 from other primate immunodeficiency viruses such as SIV_{MAC}. HIV-1 also differs from SIV_{MAC} in its sensitivity to a non-immunosuppressive cyclophilin-binding drug, suggesting that the antiviral activity of the drug is related to its effect on the p55^{gag}-cyclophilin A interaction. Our results indicate that the selective interaction between the cytosolic peptide-prolyl *cis-trans* isomerase and p55^{gag}, which is transported through the cytosol¹², is not important for p55^{gag}-directed viral particle formation. But the correlation between virion-associated cyclophilin A levels and virion infectivity implies that the p55^{gag}-cyclophilin A interaction is relevant at an early step of the HIV-1 replication cycle. □

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Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification

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INWARDLY rectifying potassium channels conduct ions more readily in the inward than the outward direction, an essential property for normal electrical activity^{1,2}. Although voltage-dependent block by internal magnesium ions may underlie inward rectification in some channels³⁻⁵, an intrinsic voltage-dependent closure of the channel plays a contributory, or even exclusive, role in others^{4,6-9}. Here we report that, rather than being intrinsic to the channel protein, so-called intrinsic rectification of strong inward rectifiers requires soluble factors that are not Mg^{2+} and can be released from *Xenopus* oocytes and other cells. Biochemical and biophysical characterization identifies these factors as polyamines (spermine, spermidine, putrescine and cadaverine). The results suggest that intrinsic rectification results from voltage-dependent block of the channel pore by polyamines, not from a voltage sensor intrinsic to the channel protein¹⁰.

To examine the mechanism of inward rectification, we expressed cloned strong inward rectifier HRK1 channels^{11,12} in *Xenopus* oocytes. Characteristic changes in ionic currents leading to the loss of inward rectification followed slowly after excision of membrane patches into a solution of high potassium concentration (K_{int}^+) without Mg^{2+} (Fig. 1a, b)¹³. Inward current amplitudes increased initially, then transient outward currents developed. The rate and degree of inactivation of outward currents decreased until only a partial inactivation remained. Patch current continually ran down after isolation, and experiments lasted only 2–10 minutes. At this stage, the time constant of inactivation at +50 mV ($\tau_{in,+50}$) reached values ranging from tens of milliseconds to seconds (104 ± 34 ms; $n = 27$). The residual rate of inactivation was slowest in inside-out patches retaining the least intracellular material, consistent with the loss of intrinsic rectification resulting from the slow dissipation of a soluble component from inside the patch¹³.

When rectification was removed, instantaneous current-voltage relations of HRK1 channels in inside-out patches displayed almost ohmic behaviour. Magnesium caused a fast voltage-dependent block of HRK1 channels, with an apparent dissociation constant at +50 mV of 9.2 ± 1.5 μ M ($n = 5$). However, even in Mg^{2+} -free solution, rectification could be restored by moving the patch back towards the oocyte (<500 μ m from the oocyte surface), and lost again by moving the patch away from the oocyte (Fig. 1e). This phenomenon was observed with all patches tested ($n = 36$) and with different speeds of solution flow in the chamber. Moving the patch close to the oocyte always caused a slow, single-exponential inactivation. Mg^{2+} -induced inactivation was a much faster process (Fig. 1c), suggesting that factors other than Mg^{2+} (intrinsic rectifying factors, IRFs) were being released from oocytes and were potent inactivating agents. The experiment shown in Fig. 1d confirms this. When intact oocytes were incubated briefly in K_{int}^+ solution, the aspirated solution reversibly restored rectification when diluted 10-fold and applied to inside-out patches of HRK1 channels ($n = 5$). We examined the effects of many substances that could conceivably be IRFs on

inside-out patches (including adenosine, guanosine and cyclic nucleotides, 20 amino acids, reduced glutathione, all at concentrations of 0.1–10 mM), and none caused rectification of HRK1 channels. As IRFs could be extracted from intact oocytes and other cells, we undertook initial purification and biochemical characterization (Table 1). The results suggested that IRFs are small compounds ($M_r < 700$) containing positively charged groups. IRF activity was not retarded on cation exchange columns at pH > 10, and in patch-clamp experiments, IRF-induced rectification was relieved at pH 9 ($\tau_{in,+50}$ at pH 7.3 was 11.3 ± 1.9 ms compared with 56.5 ± 11.4 ms at pH 9; $n = 4$), suggesting that the positively charged groups were basic, probably amines. Naturally occurring polyamines have all these properties and can be present in cells at several millimolar total concentrations¹⁴, although free concentrations may be much lower¹⁵. Stage V and VI *Xenopus* oocytes reportedly contain 600–700 μ M spermidine ($NH_3^+C_3H_6NH_2^+C_4H_8NH_2^+$) and putrescine ($NH_2^+C_4H_8NH_2^+$), and 100–300 μ M spermine ($NH_3^+C_3H_6NH_2^+C_4H_8NH_2^+C_3H_6NH_2^+$)¹⁶. When applied to inside-out membrane patches, micromolar spermine and spermidine caused steeply voltage-dependent rectification of HRK1 channels, with putrescine and cadaverine ($NH_2^+C_6H_{10}NH_2^+$) having less dramatic effects (Fig. 2). Related compounds— γ -aminobutyrate, α -aminobutyrate, ornithine, lysine, arginine or creatinine—were without effect. Polyamine-induced rectification was reversible, the time course of recovery being comparable to that of loss of rectification following patch excision. Fractions from

TABLE 1 Biochemical characterization of IRF activity in conditioned K_{int}^+ solution

Manipulation	Biological activity on HRK1 channels in inside-out patches
Control*	Full block of HRK1 current at +50 mV
K_{int}^+ conditioned, as below, by:	
Chicken fibroblasts (0.1 ml p.c.v.)	Full block of HRK1 current at +50 mV
Rat cardiac myocytes (0.1 ml p.c.v.)	Full block of HRK1 current at +50 mV
Heat inactivation (95 °C, 60 min)	Full block of HRK1 current at +50 mV
Two weeks at room temperature	No block of HRK1 current
Trypsin (0.1 mg ml ⁻¹ , 60 min, 20 °C)	Full block of HRK1 current at +50 mV
Protease (0.1 mg ml ⁻¹ , 60 min, 37 °C)	Full block of HRK1 current at +50 mV
Changing pH between 6.0 and 10.0	HRK1 blocking activity is lost above pH 9
G10-Sephadex column (retains $M_r < 700$)†	HRK1 blocking activity peaks in partially retained volume
C18 Sep-Pak column	HRK1 blocking activity is in flowthrough when applied in K_{int}^+ solution
S-Sepharose cation-exchange column‡	
Applied at pH 5	HRK1 blocking activity eluted at 400 mM K-acetate, pH 5
Applied at pH 10	Blocking activity not retained at pH 10

* The control manipulation consisted of 1 ml K_{int}^+ containing IRF after conditioning by 10–20 intact injected or non-injected *Xenopus* oocytes for 30 min at room temperature (see Fig. 1 legend).

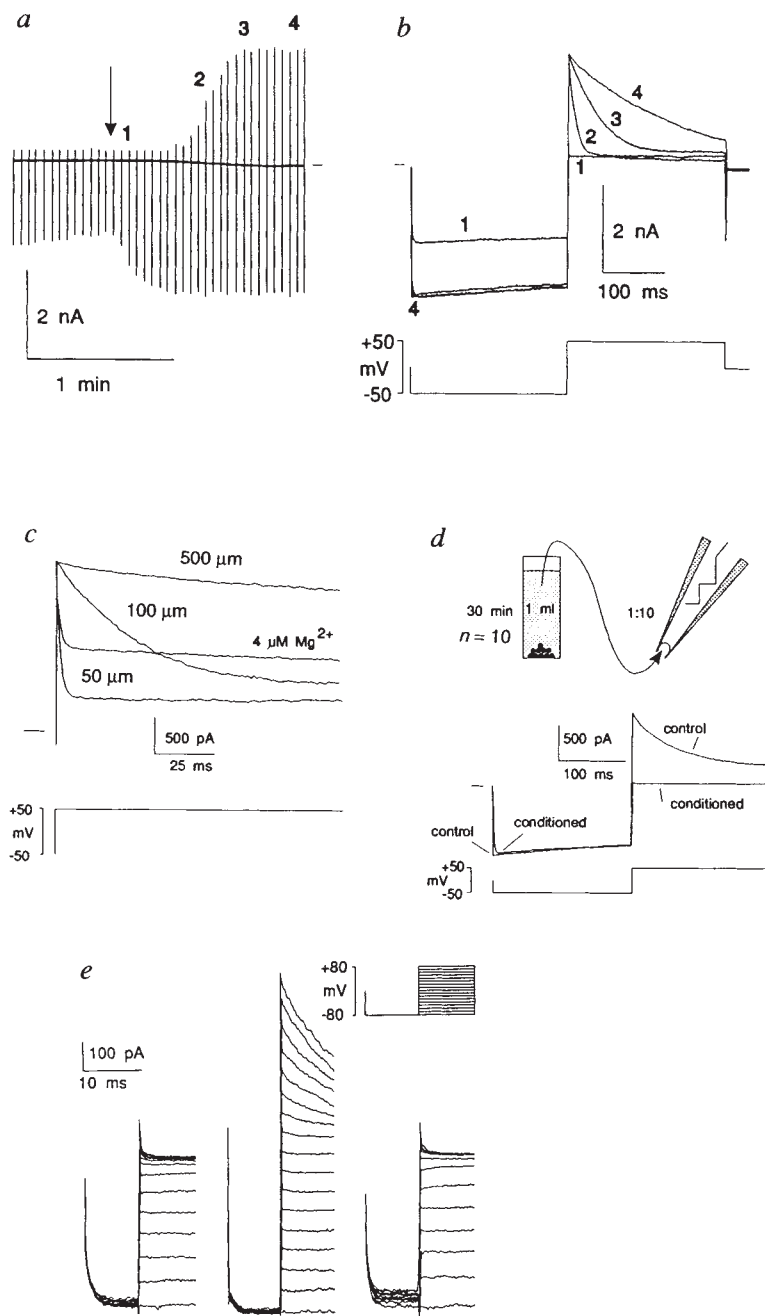
† Sephadex G-10 (M_r cut-off <700) column (15 mm $\times$ 19 cm) was equilibrated with K_{int}^+ solution before loading 1 ml of blue dextran (Sigma), or oocyte supernatant solution and was equilibrated with Na_{int}^+ (K_{int}^+ with 140 nM K^+ replaced by 140 mM Na^+) solution before loading 1 ml of K^+ -containing solution. With blue dextran and K^+ ion experiments, the effluent was collected in 1.3-ml fractions and 3-ml fractions with oocyte supernatant. With blue dextran fractions, transmittance was measured with a UV-1201 (Shimadzu, Kyoto) spectrophotometer (at 500 nm wavelength). Potassium concentration in eluate fractions was measured using a K^+ -selective electrode. The physiological effect of the eluate was estimated from the time constant of current inactivation at +50 mV.

‡ Partially purified IRF was applied to a S-Sephadex cation exchanger after a G-10 column (Sephadex C25; Sigma), with bed volume 5 ml at pH 7.3, and eluted by application of a linear potassium acetate gradient at pH 5 at a flow rate of 2 ml min⁻¹. 5-ml fractions were collected and conductivity was adjusted to that of K_{int}^+ solution by dilution with (mM) HEPES (10), EGTA (1), EDTA (1), buffer and the pH readjusted to 7.3 before application to inside-out membrane patches.

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FIG. 1 a, Slow time base HRK1 current records in response to voltage steps from zero to -50 mV and then to $+50$ mV before and after patch excision (at the arrow). Interpulse duration was 3 s. b, Fast time base records of currents taken from a as indicated. Zero current is indicated by a dash. c, Currents in response to voltage steps from -50 mV to $+50$ mV after patch isolation, with the patch placed at indicated distances from the oocyte surface, or at a large distance from the oocyte, in the presence of $4 \mu\text{M}$ free Mg . d, Currents in response to voltage steps from 0 mV holding potential to -80 mV and then to voltages between -80 and $+80$ mV, on-cell (left), inside-out (3 min after patch isolation, centre) and after subsequently approaching the oocyte ($<20 \mu\text{m}$, right).

METHODS. Complementary RNAs were transcribed *in vitro* from linearized template cDNAs using T3 or T7 RNA polymerase and the cap analogue diguanosine triphosphate¹². Stage V-VI *Xenopus* oocytes were isolated by partial ovariectomy under tricaine anaesthesia. Oocytes were then defolliculated by treatment with 1 mg ml^{-1} collagenase (Sigma type 1A) in zero Ca^{2+} ND96 solution (containing (in mM): NaCl 96, KCl 2, MgCl_2 1, HEPES 5; pH 7.5) for 1–2 h. Between 2 and 24 h after defolliculation, oocytes were pressure-injected with $\sim 50 \text{ nl}$ of 1 – $100 \text{ ng } \mu\text{l}^{-1}$ cRNA. Oocytes were maintained in 1.8 mM Ca^{2+} ND96 supplemented with penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$) for 2–7 days before experimentation. Oocytes were placed in a hypertonic solution (in mM: KCl 60, EGTA 10, HEPES 40, sucrose 250, MgCl_2 8; pH 7.0) for 5–30 min to allow separation of the oocyte membrane from the vitelline membrane; the latter was then mechanically removed. Oocytes were patch-clamped using micropipettes pulled from thin-walled glass (WPI, New Haven, CT) on a horizontal puller (Sutter Instruments, Novato, CA). Electrode resistance was typically 0.5 – $2 \text{ M}\Omega$ when filled with 140 mM KCl or NaCl, and electrode tip diameter was 5 – $15 \mu\text{m}$. Membrane patches were voltage-clamped using an Axopatch 1B patch-clamp (Axon Instruments, Foster City, CA) in a chamber mounted on the stage of an inverting microscope (Nikon Diaphot; Nikon, Garden City, NY). Most experiments were done at room temperature. In some cases, the bath temperature was lowered to 12 – 15°C as this slowed the rate of rundown of HRK1 channels. PClamp software and a Labmaster TL125 D/A converter (Axon) were used to generate voltage pulses. Data were normally filtered at 0.5 – 3 kHz , without correction for leakage or capacitive currents, digitized at 22 kHz (Neurocoder, Neurodata, NY) and stored on video tape. Experiments were replayed onto a chart recorder, or digitized into a microcomputer using Axotape software (Axon). Standard pipette (extracellular) and bath (intracellular) solutions (K_{int}^+) had the following composition (mM): KCl 140, K-HEPES 10, K-EGTA 1, K-EDTA 1. The solution pH was 7.3. EDTA was omitted from the pipette solution. Additions or alterations are noted in other figures. For on-cell patch recordings, oocyte resting potential was brought to ~ 0 by K_{int}^+ solution in the bath. In d, oocyte conditioned solution was obtained as follows and then diluted 10-fold



in K_{int}^+ . Intact defolliculated oocytes (10–20, injected or non-injected) were washed for 1 min in K_{int}^+ , incubated, without stirring, at room temperature in K_{int}^+ for 0.5–3 hours, and then the conditioned solution was aspirated and filtered through a $0.22\text{-}\mu\text{m}$ filter and stored at $+4^\circ\text{C}$.

S-Sepharose exchange columns were dansylated and separated using C_{18} high-performance liquid chromatography¹⁷. Naturally occurring polyamines were not detected in inactive fractions, but biologically active fractions contained putrescine and spermidine and lower amounts of spermine (relative peak heights of putrescine:spermidine:spermine were 34:9:1). Spermine and spermidine are, respectively, about 100- and 10-fold more potent blockers of HRK1 channels than putrescine or Mg^{2+} (Fig. 2e), and are likely to be the major IRFs in oocytes. It is known that Mg^{2+} block of inward rectifiers is not as steep as the measured rectification in intact cells¹. Spermine and spermidine block was

considerably steeper than the block by Mg^{2+} (Fig. 2d). Because closely related compounds containing carboxyl groups or ring structures did not induce rectification, we suggest that linear polyamines can pass deep into the narrow channel pore to reach the blocking site. This hypothesis¹⁸ is supported by our finding that the steepness of the voltage dependence of the block increases with the polyamine charge (Fig. 2d).

Inside-out membrane patches from oocytes expressing the strong inward rectifier IRK1 also showed slow loss of intrinsic rectification following patch excision which was restored by exposure to spermidine ($n=3$). Both ROMK1¹⁹ and delayed

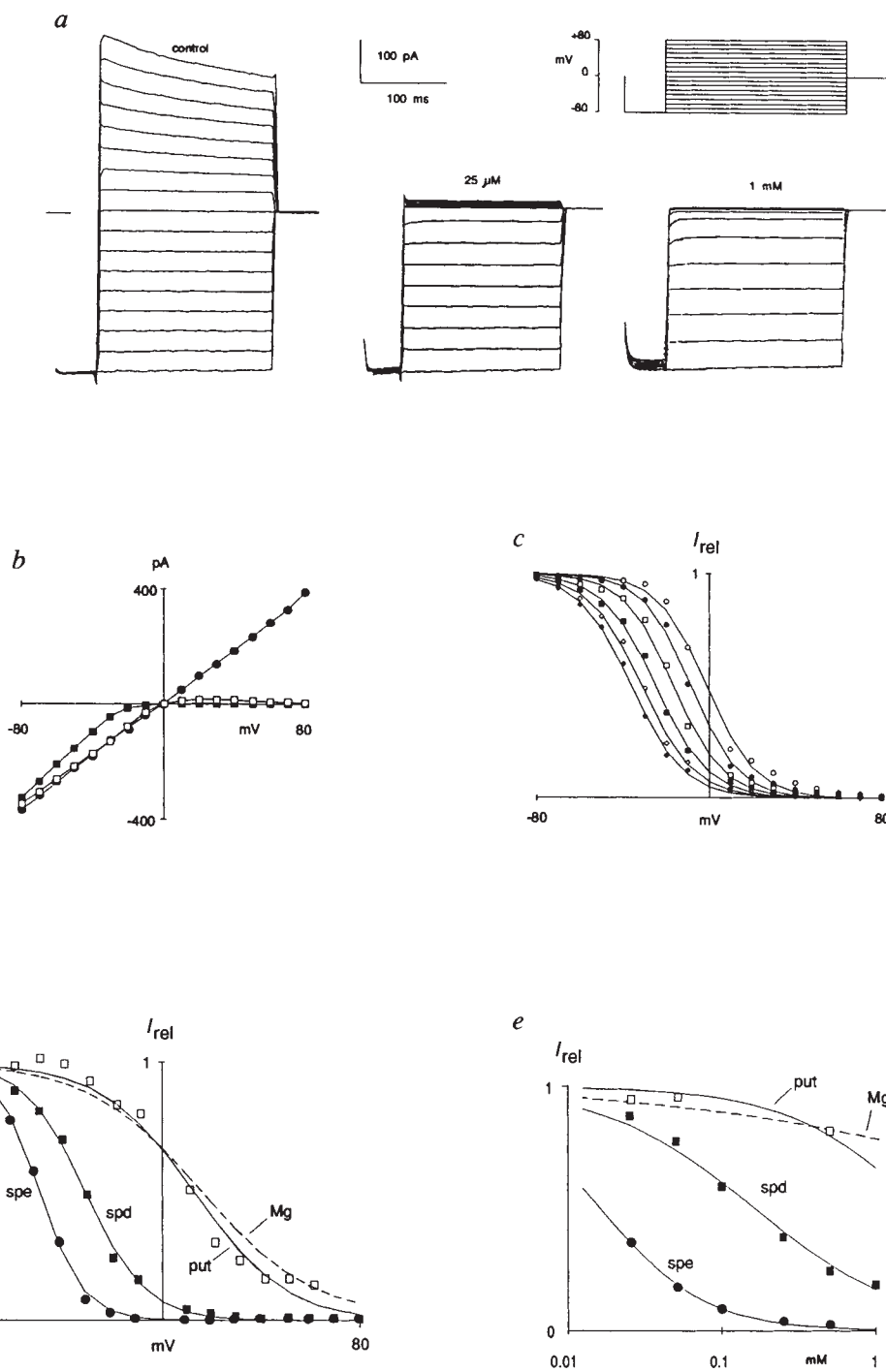


FIG. 2 *a*, HRK1 current records in response to voltage steps between -80 and $+80$ mV, 3 min after patch isolation (left) and in the presence of $25 \mu\text{M}$ (centre) and 1 mM spermidine (right). To avoid slow deactivation at negative voltages, patches were held at 0 mV and briefly stepped to -80 mV (5 ms) before stepping to the test voltage. *b*, Currents at the end of 20 ms test-pulse versus voltage, after patch isolation ($\bullet$) and in the presence of $25 \mu\text{M}$ ($\square$) and 1 mM ($\blacksquare$) spermidine. *c*, Current in spermidine ($\circ$, $25 \mu\text{M}$; $\bullet$, $50 \mu\text{M}$; $\square$, $100 \mu\text{M}$; $\blacksquare$, $250 \mu\text{M}$; $\diamond$, $500 \mu\text{M}$; $\blacklozenge$, 1 mM), relative to peak outward current before spermidine, (I_{rel}) versus membrane potential. Curves are fitted with Boltzmann functions using $Z = 2.2$ – 2.3 . *d*, Current in $500 \mu\text{M}$ spermine (spe), spermidine (spd) and putrescine (put) relative to control (I_{rel}) versus membrane potential, from a representative patch. Curves are fitted with Boltzmann functions. Average values for Z ($=$ charge $\times$ electrical distance) were 2.64 ± 0.01 (spe; $n = 5$), 2.21 ± 0.03 (spd; $n = 6$), 1.69 ± 0.15 (put; $n = 4$) and 1.12 ± 0.10 (Mg^{2+} ; $n = 5$). The dashed line is the predicted relationship for Mg^{2+} . *e*, Dose-response curve for current in spermine (spe), spermidine (spd) and putrescine (put) relative to control (I_{rel}) at -20 mV, for the same patch as in *d*. Curves are fitted with the Hill equation, using $H = 1$. Dashed line indicates predicted Mg^{2+} dependence.

outward rectifier DRK1 [$\text{Kv}2.1$]²⁰ channels showed only mild inward rectification (Fig. 3*a*). Neither bringing patches containing these channels close to the surface of oocytes, nor bath application of oocyte-conditioned K^+_{int} induced strong rectification. In contrast to the submicromolar sensitivity of HRK1 channels to spermine at positive voltages, $50 \mu\text{M}$ spermine had no effect on DRK1 patch currents at $+50$ mV ($n = 5$; Fig. 3*b*), and 1 mM spermine inhibited DRK1 channels by only $45 \pm 5\%$ at $+50$ mV. Both DRK1 and ROMK1 channels have slightly voltage-dependent, millimolar, sensitivity to block by magnesium^{21,22}. Thus, high polyamine sensitivity is only seen with strong inward rectifier channels (IRK1, HRK1), and parallels sensitivity to block by Mg^{2+} . It seems likely that Mg^{2+} and polyamines share common binding sites^{23–25}, and in IRK1 channels both Mg^{2+} affinity and 'intrinsic' rectification depend on the presence of a

negatively charged residue in transmembrane domain M2 (refs 26, 27). The introduction of a negative charge at this site (N171D; one-letter amino-acid code) into ROMK1 converted the channel into a strong inward rectifier²⁸, and induced a high sensitivity to block by internal spermidine (Fig. 3*c*).

We have shown that 'intrinsic' rectification results from block by cytoplasmic factors, not from a voltage-sensor within the channel protein, and that these factors are likely to be polyamines. Some of the diverse biological effects of polyamines may be related to induction of intrinsic rectification in potassium channels. For instance, both epilepsy and cardiac hypertrophy are associated with enhanced cellular excitability and elevated polyamine levels^{29,30}. As a general concept, increasing levels of polyamines will increase the degree of rectification and increase excitability. Given the steep voltage-dependence of polyamine

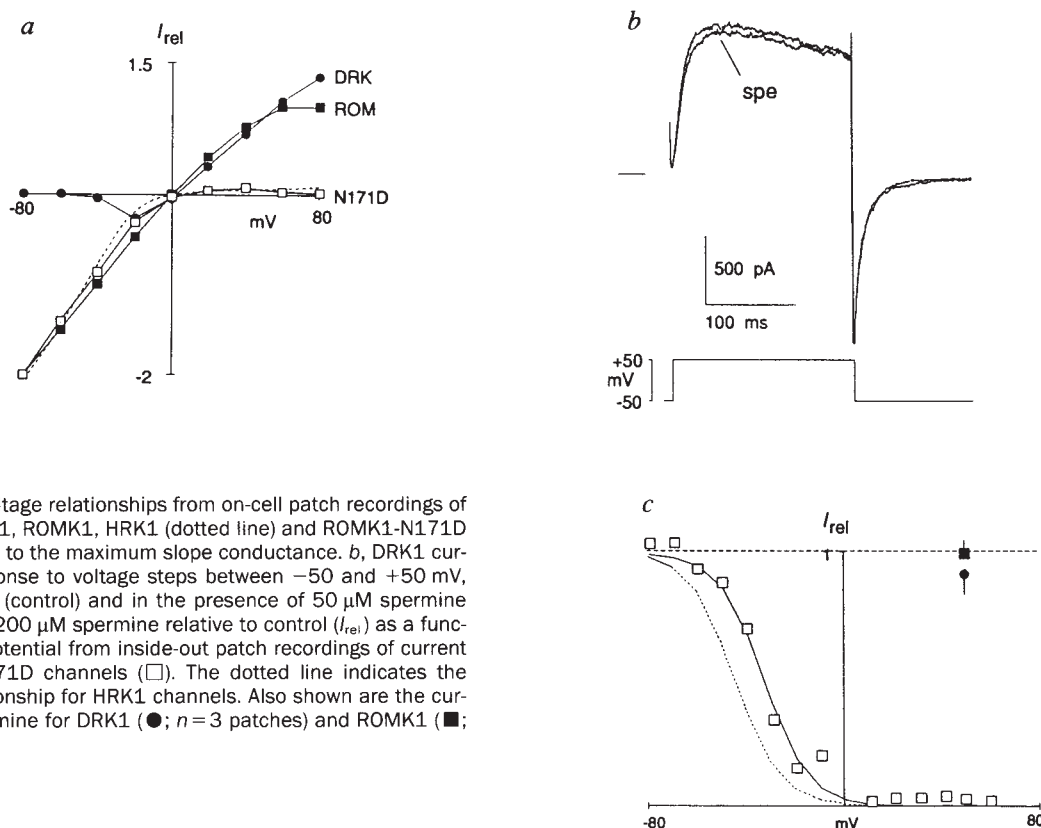


FIG. 3 *a*, Current-voltage relationships from on-cell patch recordings of current through DRK1, ROMK1, HRK1 (dotted line) and ROMK1-N171D channels normalized to the maximum slope conductance. *b*, DRK1 current records in response to voltage steps between -50 and +50 mV, after patch isolation (control) and in the presence of 50 μ M spermine (spe). *c*, Currents in 200 μ M spermine relative to control (I_{rel}) as a function of membrane potential from inside-out patch recordings of current through ROMK1-N171D channels ($\square$). The dotted line indicates the corresponding relationship for HRK1 channels. Also shown are the currents in 50 μ M spermine for DRK1 ($\bullet$; $n=3$ patches) and ROMK1 ($\blacksquare$; $n=3$) channels.

block, it is likely that even small changes of cytoplasmic polyamine levels could have significant effects on cellular excitability. $\square$

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Molecular mechanism of cyclic-nucleotide-gated channel activation

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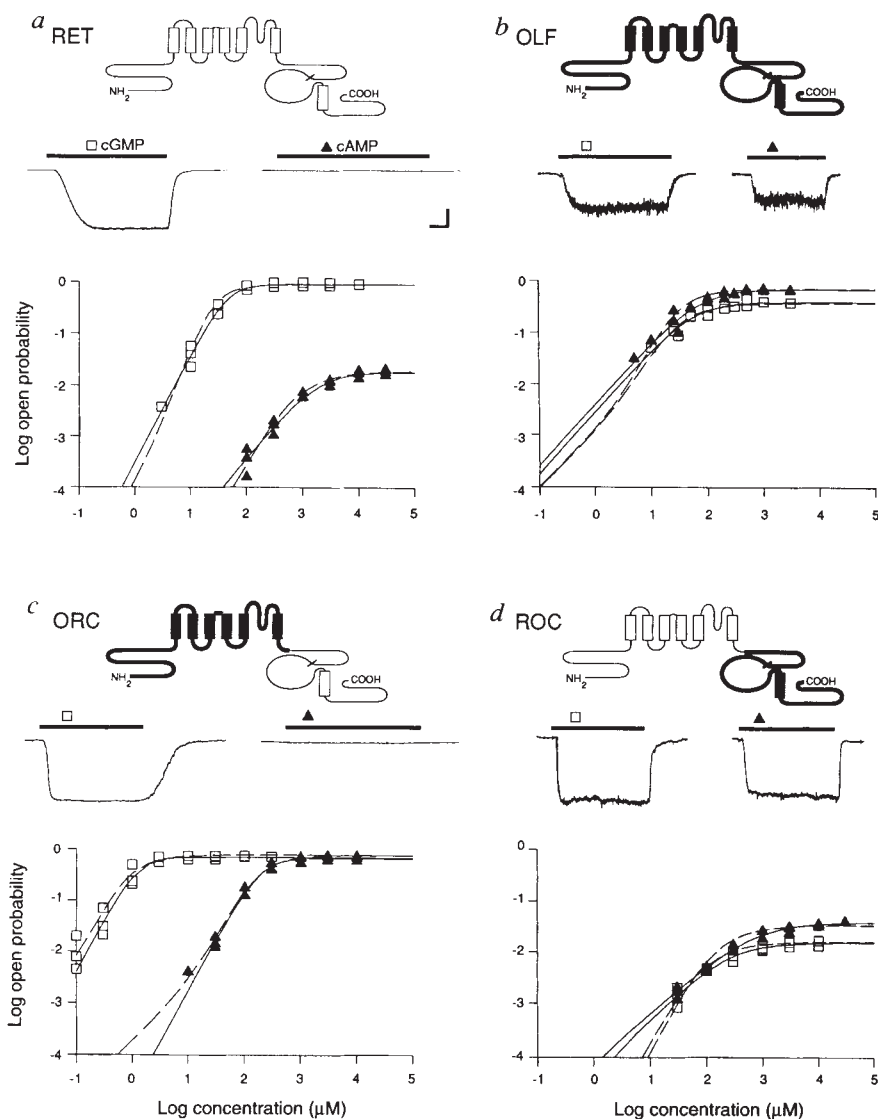
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STUDIES on the activation of ligand- and voltage-gated ion channels have identified regions involved in both ligand binding¹ and voltage sensing², but relatively little is known about how such domains are coupled to channel opening. Here we investigate the structural basis for the activation of cyclic-nucleotide-gated channels, which are directly opened by cytoplasmic cyclic nucleotides^{3,4} but are structurally homologous to voltage-gated channels⁵⁻⁷. By constructing chimeras between cyclic-nucleotide-gated channels cloned from bovine retinal photoreceptors⁸ and catfish olfactory neurons⁷, we identify two distinct domains that are important for ligand binding and channel gating. A putative α -helix in the carboxy-terminal binding domain determines the selectivity of the channel for activation by cGMP relative to cAMP. A domain in the amino-terminal region determines the ease with which channels open and thus influences agonist efficacy. We propose that channel opening is coupled to an allosteric conformational change in the binding site which enhances agonist binding. Thus, cyclic nucleotides activate the channel by binding tightly to the open state and stabilizing it.

The carboxy terminus of cyclic-nucleotide-gated (CNG) channels contains a stretch of 130 amino acids which is homologous

FIG. 1 Agonist selectivity of *a*, *b*, wild-type, and *c*, *d*, C-terminus chimaera CNG channels. Macroscopic currents and dose-response curves for cGMP (squares) or cAMP (triangles) applied to inside-out patches from *Xenopus* oocytes injected with synthetic RNAs for RET, OLF, ROC (RET with OLF C terminus) and ORC (OLF with RET C terminus). Channel open probability plotted as a function of cyclic nucleotide concentration ($n = 3$ for each channel). Solid lines are best fits of Hill equation; dashed lines are fits of Monod-Wyman-Changeux allosteric model¹⁷ (see text and Table 1). Icons represent channels with length of regions proportional to number of amino acids. First six rectangles represent S1–S6 membrane-spanning domains; rectangle in the C terminus represents the C-helix of the binding domain (see Fig. 2 and text). The binding domain's N terminus is indicated by a diagonal line; the C terminus is at end of the C-helix. Unfilled rectangles and thin lines represent RET sequences; filled rectangles and thick lines represent OLF sequences. Bars indicate perfusion with 30 μ M cyclic nucleotide. Horizontal scale bar: 5 s for RET and ROC; 10 s for OLF and ORC. Vertical scale bar: 100 pA for RET, 125 pA for ROC, 2 pA for OLF and 5 pA for ROC. Membrane potential was -80 mV.

METHODS. RNA transcribed from cDNA inserts in pGEM-3Z containing 5' and 3' *Xenopus* β -globin untranslated sequences flanking the polylinker^{24,27}. Chimaeric PCR products were generated²⁴ and subcloned into cDNA insert using available restriction sites. Correct chimaeric cDNA was verified by double-stranded dideoxy chain-termination sequencing across the entire fragment amplified by PCR and the restriction sites used for subcloning. In ROC, amino acids N423–D690 of RET were replaced by K393–T682 of OLF. In ORC, K393–T682 of OLF were replaced by N423–D690 of RET. Patch clamp recordings were made as described²⁴. For dose-response curves,



currents were normalized to maximum response (at saturating agonist concentrations) and converted to open probability (see Table 1).

to the cyclic-nucleotide-binding domains of other proteins^{7,8} (Figs 1, 2c). To determine the role of the carboxy terminus in ligand binding, we took advantage of the fact that retinal and olfactory CNG channels respond differently to cAMP and cGMP. Thus, the bovine retinal CNG channel (RET) is preferentially activated by cGMP^{7,8}, having a 30-fold higher apparent affinity (from $K_{1/2}$ values) and a 45-fold greater efficacy (determined by the maximal open probability, p_{max} , at saturating ligand concentrations) for cGMP than for cAMP. In contrast, the non-selective catfish olfactory CNG channel (OLF) displays similar $K_{1/2}$ and p_{max} values in response to cGMP or cAMP (Fig. 1 and Table 1). Two complementary chimaeras, ROC (retinal channel with an olfactory channel C terminus) and ORC (olfactory channel with a retinal channel C terminus) demonstrate that the cyclic nucleotide selectivity of the channels is largely determined by the C terminus (Fig. 1). Thus, ROC resembles the olfactory channel because it is activated equally by cAMP and cGMP, whereas ORC resembles the retinal channel in that it is selectively activated by cGMP (all at 30 μ M; Fig. 1). Dose-response curves for ROC show that its $K_{1/2}$ and p_{max} values for cGMP and cAMP are similar to each other, resembling the behaviour of the olfactory channel (Fig. 1b and Table 1). Con-

versely, ORC resembles the retinal channel in that it displays a much higher apparent affinity for cGMP than for cAMP.

We compared the agonist selectivity of the channels quantitatively using a scheme first proposed for the nicotinic ACh receptor⁹: $A + R \xrightleftharpoons{K} AR \xrightleftharpoons{L} AR^*$, where R is the closed unliganded channel, A is an agonist molecule, AR is a closed liganded channel and AR^* is an open liganded channel. The total free energy change of activation is the sum of the free energy changes associated with binding (K) and gating (L): $\Delta G = RT \ln(KL) = -RT \ln(p_{max}/K_{1/2})$ (see Fig. 2 legend). The agonist selectivity of a channel was then defined as the difference in ΔG between cGMP and cAMP: $\Delta(\Delta G) = \Delta G_{cGMP} - \Delta G_{cAMP}$ (refs 10–13). The $\Delta(\Delta G)$ values confirm that the retinal channel is highly selective for cGMP ($\Delta(\Delta G) \ll 0$), whereas the olfactory channel is non-selective ($\Delta(\Delta G) = 0$). The selectivity of ORC resembles that of the retinal channel; the selectivity of ROC resembles the olfactory channel (Fig. 2a). A model with four binding sites gives similar results (Fig. 2; Table 1). Thus, the major determinates of cyclic-nucleotide binding are in the C terminus.

We next localized the C-terminal domain that governs agonist selectivity (Fig. 2b, c) by focusing on the 130-amino-acid region of homology between the CNG channels and the cAMP-binding

domain of the catabolite-gene-activator protein (CAP), whose structure has been solved¹⁴. The importance of this region was demonstrated by the chimaera ROCB, in which the selectivity of the olfactory channel was conferred onto the retinal channel by exchanging only these 130 amino acids (Fig. 2b). The cAMP-binding domain of CAP consists of an N-terminal α -helix (the A-helix) preceding an eight-stranded β -roll, followed by two α -helices (B and C). The C-helix makes two hydrogen bonds with the C6-amino group of the purine ring of cAMP¹⁴. Because cAMP and cGMP only differ in their purine ring substitutions, a region homologous to the C-helix may specify agonist selectivity in the CNG channels. Accordingly, we exchanged only the putative C-helices (24 amino acids) between the retinal and olfactory channels (ROC_a, ORC_a). We find the C-helix is both necessary (ROCD: ROCB without the olfactory C-helix) and sufficient to determine agonist selectivity (Fig. 2b). Given the homology between the binding domains of the CNG channels and protein kinases¹⁵, the C-helix may play a general role in determining ligand specificity.

Although cyclic nucleotide selectivity localizes to the C-helix of the C terminus, the N terminus and transmembrane regions of the channel strongly influence efficacy and the absolute values of agonist affinity (Fig. 1). Thus, the $p_{\max}$ elicited by cAMP or cGMP in ROC (~ 0.03) is much lower than in the olfactory channel ($p_{\max} \approx 0.5$). Conversely, the $p_{\max}$ with cAMP in ORC (0.7) is much greater than it is in the retinal channel (0.02) and equal to the $p_{\max}$ with cGMP. Finally, the absolute agonist

affinity of ROC is reduced relative to the olfactory channel, whereas the affinity of ORC for cGMP is greatly enhanced compared to the retinal channel.

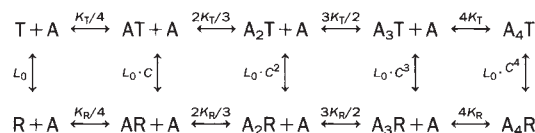
To localize the regions of the olfactory channel responsible for these differences in the activation properties of ORC and the retinal channel, we replaced segments of the amino-terminal and transmembrane region of the retinal channel with sequences from the olfactory channel (Fig. 3). These experiments identify a new domain, which we term N-S2, which is responsible for both the enhanced efficacy of cAMP and greater affinity for cGMP in ORC relative to the retinal channel. The N-S2 domain starts in the middle of the cytoplasmic portion of the N terminus and extends through the intracellular loop connecting the S2 and S3 transmembrane helices (Fig. 3, RON-S2 chimaera). Swaps of smaller regions produce a correspondingly smaller enhancement of cAMP efficacy. Surprisingly, given the proposed role of S4 as the voltage sensor² and the P-domain as the channel pore¹⁶, swaps of these regions do not increase the efficacy of cAMP (chimaera ROSB; see Fig. 3 legend).

These results predict that replacing both the N-S2 domain and C-helix of the olfactory channel with the corresponding regions from the retinal channel (ORN-S2C_a) should completely convert the activation properties of the olfactory channel to those of the retinal channel. A complementary chimaera, RON-S2C_a, should convert the activation properties of the retinal channel to those of the olfactory channel. These predictions are largely borne out (Fig. 4a). Thus, the gating of ORN-S2C_a resembles

TABLE 1 Fitted dose-response curve parameters

	Hill equation parameters						MWC model parameters				
	$K_{1/2}$ (μ M)		n		$p_{\max}$		K_T (μ M)		K_R (μ M)		L_0
	cGMP	cAMP	cGMP	cAMP	cGMP	cAMP	cGMP	cAMP	cGMP	cAMP	
RET*	63	1,968	2.0	1.2	0.9	0.02	33.0	330	1.0	50	100,000
OLF†	73	65	1.2	1.5	0.4	0.6	17.0	25	3.0	4	1,000
ROC	250	614	1.0	1.0	0.02	0.04	40.0	65	6.0	8	100,000
ORC	1	227	1.7	1.8	0.7	0.7	0.8	150	0.1	20	1,000

Values are best fits of the Hill equation or Monod-Wyman-Changeux (MWC) allosteric model¹⁷ to the data of Fig. 1. Hill equation: $R/R_{\max} = 1/(1 + (K_{1/2}/[A])^n)$, where $[A]$ is the cyclic nucleotide concentration, and n is the Hill coefficient. For the allosteric scheme we assumed that the channel exists in either a closed (T) or open (R) state. The tetrameric channel¹⁸ contains four independent binding sites with distinct dissociation constants for closed (K_T) and open (K_R) states. Each successive binding of agonist (A) causes a progressive shift in the equilibrium towards the open state, given by the factor $C = K_R/K_T$:



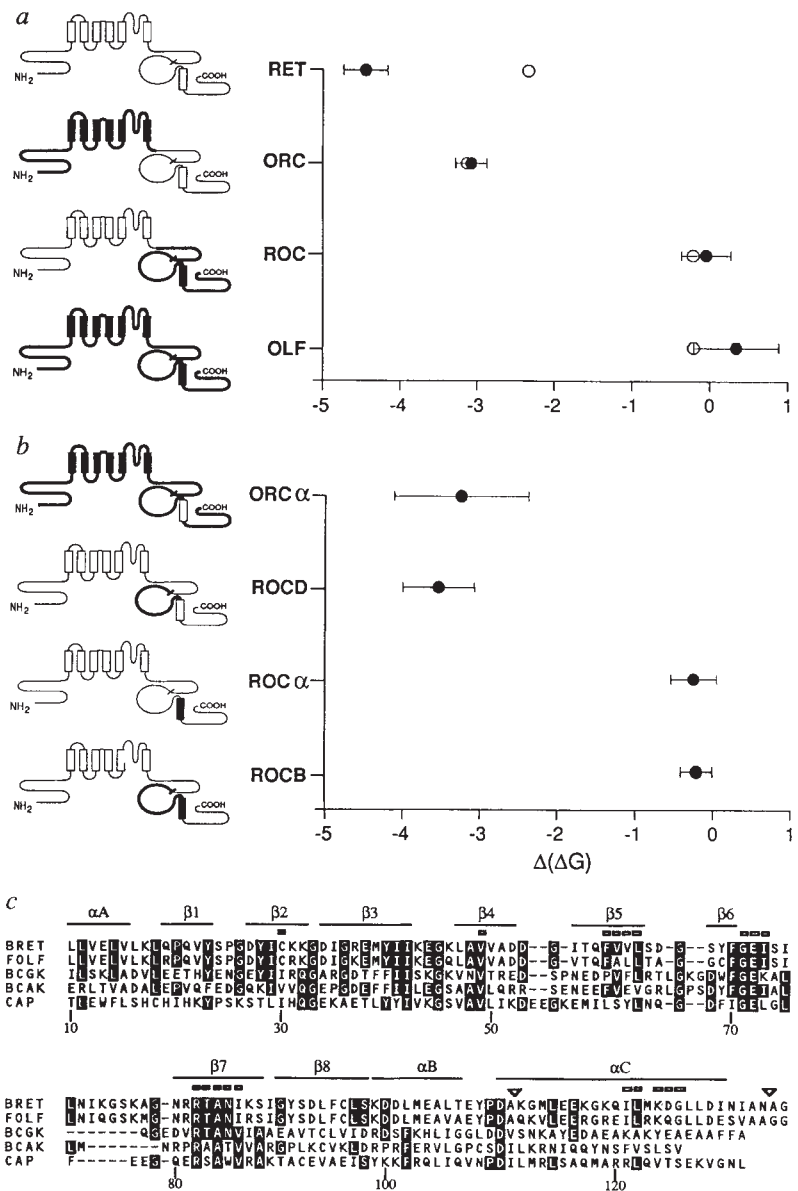
Channel open probability (p_o) predicted by this scheme is given by: $p_o = (1 + \alpha)^n / [L_0(1 + C\alpha)^n + (1 + \alpha)^n]$, where $\alpha = [A]/K_R$, $L_0 = [T]/[R]$, $C = K_R/K_T$, $n = 4$ (number of independent binding sites). L_0 was fixed as 1,000 for OLF and 100,000 for RET, reflecting the hypothesis that OLF is more readily opened. L_0 for ORC and ROC were then set to the values in OLF and RET, respectively, according to our hypothesis that the N-S2 domain determines the ease of channel opening. We then fitted this equation to the dose-response data of Fig. 1 by adjusting K_R and K_T (Fig. 1). The fits are not unique and are only intended to show that the model is consistent with the data. To measure p_o from the dose-response data (see Fig. 1, for example), we first used 'noise' analysis to measure $p_{\max}$ at saturating concentrations of cyclic nucleotide. Power density spectra were calculated from 5–10 s of data in the presence or absence of cyclic nucleotide (low-pass filtered at 3.5 kHz or 0.45 kHz; digitized at 8.192 kHz or 1.024 kHz). Difference spectra were fitted with two Lorentzian components, and variance (σ^2) calculated from the area under the Lorentzians. Assuming binomial statistics, $p_{\max} = 1 - [\sigma_{\max}^2 / I_{\max}] / [\sigma_{\text{low}}^2 / I_{\text{low}}]$, where $\sigma_{\max}^2$ and σ_{low}^2 are variances at saturating and low concentrations of agonist, respectively; $I_{\max}$ and I_{low} are corresponding macroscopic currents. Normalized dose-response curves were multiplied by $p_{\max}$ to obtain p_o . This technique works for values of $p_{\max} > 0.2$ and gives results in agreement with previous single-channel measurements^{7,24}. For ROC and ROC_a, where $p_{\max} < 0.1$, we relied on the voltage-dependent open channel block produced by the Shaker N-terminal inactivation ball peptide^{25,26}. At saturating cyclic nucleotide, a high concentration of ball peptide was applied at +100 mV to drive channels into the open-blocked state. Upon repolarization to -80 mV (where there is little block), a large tail current is observed whose rising phase reflects rapid unblock and whose falling phase reflects slower re-equilibration as excess open channels close²⁶. The ratio of the CNG current at -80 mV (no peptide) to the peak tail current provides an upper limit for $p_{\max}$. To determine $p_{\max}$ more precisely, tails were fitted by two exponential functions according to a simple open-channel block scheme²⁶. This method was verified using the cAMP response of RET, where $p_{\max}$ could be independently determined from the ratio of maximal responses to cAMP to cGMP.

* RET, retinal channel.

† OLF, olfactory channel.

FIG. 2 Cyclic nucleotide selectivity of CNG channels defined by $\Delta(\Delta G)$. **a**, Comparison of RET, OLF, ROC and ORC. Solid circles, $\Delta(\Delta G)$ values from del Castillo and Katz scheme; open circles, $\Delta(\Delta G)$ values from Monod-Wyman-Changeux (MWC) allosteric model (see Methods). The differences between ORC and RET are largely model-dependent. **b**, Localization of the site of selectivity to the C-helix. ROCB, RET channel that contains the 132 amino acids of the putative OLF binding domain. ROCD, similar to ROCB but retains the RET C-helix. ORC $_{\alpha}$ and ROC $_{\alpha}$, only the 24 amino acid residues of the C-helices have been exchanged. Bars represent s.d. **c**, Amino-acid sequence alignment of the bovine RET channel (BRET) amino acid residues L485–G615⁸, catfish OLF (FOLF) residues L455–G585⁷, the bovine cGMP-dependent kinase domain RII residues I228–A352²⁸ (BCGK), and the bovine cAMP-dependent kinase domain RII residues E261–V379²⁹ (BCAK) with the cyclic-nucleotide-binding domain of CAP¹⁴. Amino-acid residue numbering of CAP is shown beneath the alignment. Lines above alignment indicate amino-acid residues that form α (A–C) helices or β (1–8)-strands in the CAP crystal structure. Squares: amino-acid residues close to cAMP bound to CAP. Triangles: region substituted in the C-helix chimaeras.

METHODS. ROCB, amino acids Q494–N613 of RET replaced by amino acids R464–A583 of OLF. (cGMP: $K_{1/2} = 255(\pm 68)$ μ M, Hill coefficient, $HC = 1.0(\pm 0.1)$; cAMP: $K_{1/2} = 579(\pm 56)$ μ M, $HC = 1.0(\pm 0.2)$; $I_{max}(cGMP)/I_{max}(cAMP) = 0.6(\pm 0.1)$; $n = 4$). ROCD, amino acids Q494–T584 of RET replaced by R464–A554 of OLF. (cGMP: $K_{1/2} = 188(\pm 86)$ μ M, $HC = 1.5(\pm 0.2)$, $p_{max} = 0.5(\pm 0.1)$; cAMP: $K_{1/2} = 932(\pm 184)$ μ M, $HC = 1.3(\pm 0.6)$, $p_{max} = 0.007(\pm 0.003)$; $n = 3$). ROC $_{\alpha}$, amino acids K590–N613 of RET replaced by Q560–A583 of OLF. (cGMP: $K_{1/2} = 516(\pm 67)$ μ M, $HC = 1.08(\pm 0.05)$, $p_{max} = 0.04(\pm 0.02)$; cAMP: $K_{1/2} = 1,240(\pm 558)$ μ M, $HC = 1.1(\pm 0.1)$, $p_{max} = 0.06(\pm 0.01)$; $n = 3$). ORC $_{\alpha}$, amino acids Q560–A583 of OLF replaced by K590–N613 of RET (cGMP: $K_{1/2} = 2(\pm 2)$ μ M, $HC = 2.0(\pm 0.5)$, $p_{max} = 0.75(\pm 0.06)$; cAMP: $K_{1/2} = 178(\pm 98)$ μ M, $HC = 1.8(\pm 0.2)$, $p_{max} = 0.4(\pm 0.1)$; $n = 4$ (3 for p_{max})). $\Delta(\Delta G)$ values for del Castillo and Katz⁹ scheme (filled circles) calculated as follows: dissociation constant determined from: $K = K_{1/2}/(1 - p_{max})$. Equilibrium gating constant determined from: $L = (1 - p_{max})/p_{max}$. Free energy of binding: $\Delta G_B = -RT \ln(1/K) = -RT \ln\{(1 - p_{max})/p_{max}\}$. Free energy of opening: $\Delta G_O = -RT \ln(1/L) = -RT \ln\{p_{max}/[1 - p_{max}]\}$. Thus, total free energy change is: $\Delta G = \Delta G_B + \Delta G_O = -RT \ln(p_{max}/K_{1/2})$. Indeterminate errors (s.d.) were propagated according to ref. 30. $\Delta(\Delta G)$ for MWC model



calculated at saturating concentrations of cyclic nucleotide when all channels exist as either T₄ or R₄. Activation analysed along one path from T to T₄ at equilibrium path is arbitrary: $\Delta G = \Delta G_B + \Delta G_O = -RT \ln\{[1/K_T]^n - \ln\{1/[L_O(K_R/K_T)^n]\}\}$; $\Delta(\Delta G) = -RT \ln(K_{RCAMP}/K_{RCGMP})$. No s.d.s are given for $\Delta(\Delta G)$ values for MWC model as they were calculated from a single value of K_R determined by fitting three sets of data for each channel by eye.

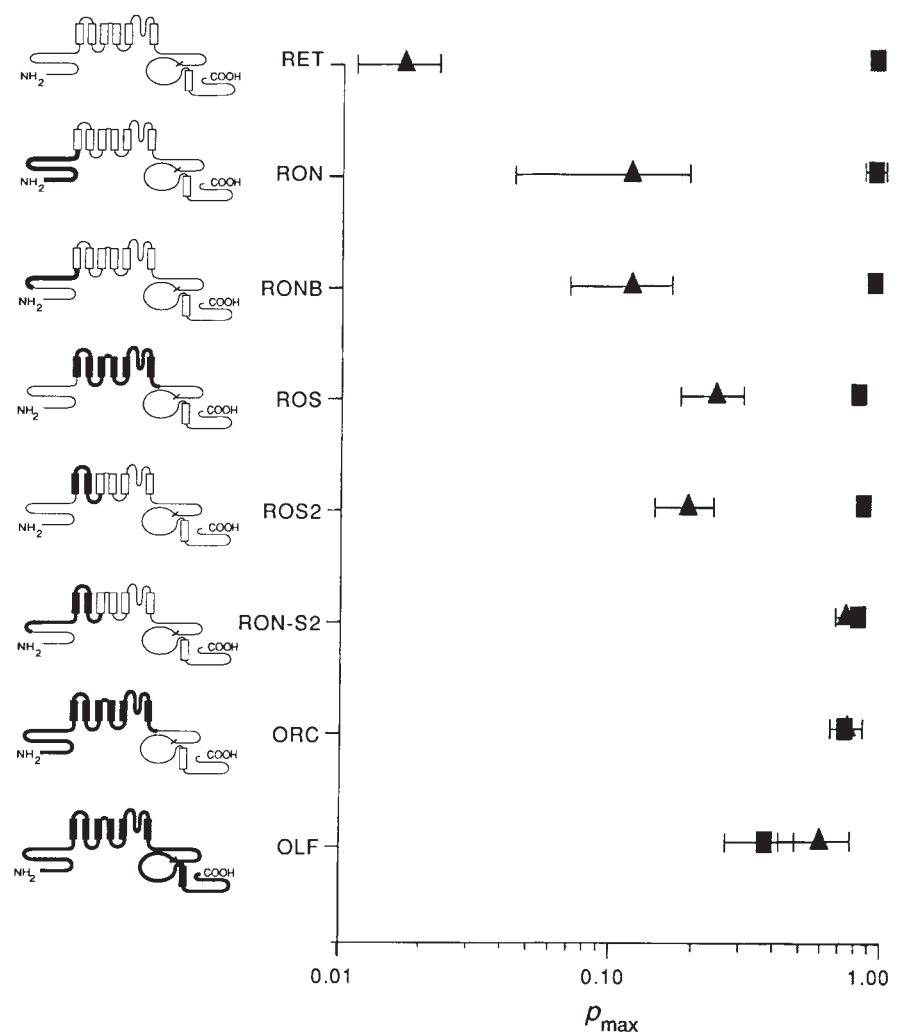
that of the retinal channel as the chimaera now exhibits the appropriately high cGMP selectivity of the retinal channel in terms of both a higher apparent affinity for and a much greater efficacy of cGMP relative to cAMP. This double chimaera also has a $K_{1/2}$ of 39 μ M for cGMP, which is now closer to the $K_{1/2}$ of the retinal channel (63 μ M) than to that of ORC $_{\alpha}$ (2 μ M). Conversely, the gating of RON-S2C $_{\alpha}$ closely resembles that of the olfactory channel as both cGMP and cAMP gate this chimaera with a high p_{max} (~0.6), similar to the p_{max} of the olfactory channel and much greater than that of ROC $_{\alpha}$ (~0.05). Although these double chimaeras do not completely mimic the wild-type channels (absolute p_{max} values in ORN-S2C $_{\alpha}$ were slightly lower than in the retinal channel and $K_{1/2}$ values in RON-S2C $_{\alpha}$ were higher than in the olfactory channel; Fig. 4a), they do confirm

that the N-S2 and C-helix domains are major determinants of CNG channel activation.

How does the N-S2 domain cause the reciprocal changes in agonist efficacy in ROC and ORC? We propose that the N-S2 domain determines the ease with which channels open (namely the free energy difference between closed and open states), with the retinal N-S2 domain rendering channels more difficult to open than the olfactory domain. Presumably agonists activate the channel by coupling a favourable free energy change of binding to an unfavourable free energy change of opening. As the p_{max} elicited by cGMP or cAMP in the olfactory channel is lower than the p_{max} with cGMP in the retinal channel, the binding of cyclic nucleotides to the olfactory channel must impart a smaller free energy change for activation compared

FIG. 3 Influence of amino transmembrane domain on $p_{\max}$. Squares, cGMP; triangles, cAMP; error bars are standard deviations. RON, entire cytoplasmic portion of N terminus of RET replaced by sequences of OLF. RONB, approximately half of the N terminus of RET closest to S1 replaced by OLF. ROS, S1–S6 transmembrane region of RET replaced by OLF. ROS2, S1, S2 and cytoplasmic loop connecting S2 to S3 of RET replaced by same region of OLF. RON-S2, region of RET from middle of N terminus through S2–S3 loop replaced by OLF. Dose-response data for these and various other chimaeras are listed below.

METHODS. RON, amino acids M1–D157 of RET replaced by amino acids M1–S132 of OLF (cGMP: $K_{1/2} = 22(\pm 11) \mu\text{M}$, $\text{HC} = 2.8(\pm 0.5)$, $p_{\max} = 0.92(\pm 0.08)$; cAMP: $K_{1/2} = 1,853(\pm 278) \mu\text{M}$, $\text{HC} = 1.6(\pm 0.1)$, $p_{\max} = 0.11(\pm 0.07)$; $n = 5$ (3 for $p_{\max}$)). RONA, M1–N88 of RET (first half of cytoplasmic N terminus) replaced by M1–S86 of OLF (cGMP: $K_{1/2} = 140(\pm 34) \mu\text{M}$, $\text{HC} = 2.0(\pm 0.7)$ ($n = 3$), $p_{\max} = 0.6$ ($n = 2$); cAMP: $p_{\max} = 0.009$ ($n = 2$)). RONB, N91–D157 of RET replaced by E89–S132 of OLF (cGMP: $K_{1/2} = 16(\pm 6) \mu\text{M}$, $\text{HC} = 2.0(\pm 0.6)$, $p_{\max} = 0.91(\pm 0.01)$; cAMP: $K_{1/2} = 2,780(\pm 870) \mu\text{M}$, $\text{HC} = 1.3(\pm 0.1)$, $p_{\max} = 0.12(\pm 0.05)$; $n = 4$ (3 for $p_{\max}$)). ROS, N161–Q417 of RET replaced by D136–H387 of OLF (cGMP: $K_{1/2} = 17(\pm 9) \mu\text{M}$, $\text{HC} = 2.0(\pm 0.3)$, $p_{\max} = 0.80(\pm 0.04)$; cAMP: $K_{1/2} = 2429(\pm 484) \mu\text{M}$, $\text{HC} = 1.5(\pm 0.2)$, $p_{\max} = 0.2(\pm 0.1)$; $n = 6$ (3 for $p_{\max}$)). ROS2, N161–S240 of RET replaced by D136–R215 of OLF (cGMP: $K_{1/2} = 18(\pm 4) \mu\text{M}$, $\text{HC} = 2.5(\pm 0.4)$, $p_{\max} = 0.84(\pm 0.01)$; cAMP: $K_{1/2} = 2,373(\pm 150) \mu\text{M}$, $\text{HC} = 1.6(\pm 0.1)$, $p_{\max} = 0.21(\pm 0.05)$; $n = 5$ (3 for $p_{\max}$)). ROSA1, N161–Y265 of RET (S1–S3) replaced by D136–V239 of OLF (cGMP: $K_{1/2} = 13(\pm 2) \mu\text{M}$, $\text{HC} = 2.3(\pm 0.4)$, $p_{\max} = 0.85(\pm 0.03)$; cAMP: $K_{1/2} = 2,326(\pm 370) \mu\text{M}$, $\text{HC} = 1.5(\pm 0.1)$, $p_{\max} = 0.2(\pm 0.1)$; $n = 6$ (3 for $p_{\max}$)). ROSA2, N161–V215 of RET (S1 and S2) replaced by D136–I190 of OLF (cGMP: $K_{1/2} = 34(\pm 8) \mu\text{M}$, $\text{HC} = 2.2(\pm 0.1)$; cAMP: $K_{1/2} = 3,320(\pm 736) \mu\text{M}$, $\text{HC} = 1.3(\pm 0.2)$; $I_{\max}(\text{cGMP})/I_{\max}(\text{cAMP}) = 20(\pm 4)$; $n = 4$). ROSA3, E189–S240 of RET (S2 and cytoplasmic loop from S2 to S3) replaced by Q164–R215 of OLF (cGMP: $K_{1/2} = 27(\pm 7) \mu\text{M}$, $\text{HC} = 2.3(\pm 0.5)$; cAMP: $K_{1/2} = 3,867(\pm 791) \mu\text{M}$, $\text{HC} = 1.2(\pm 0.1)$, $I_{\max}(\text{cGMP})/I_{\max}(\text{cAMP}) = 13(\pm 6)$; $n = 5$). ROSB, E267–Q417 of RET (S4–S6) replaced by Q241–H387 of



OLF (cGMP: $K_{1/2} = 70(\pm 36) \mu\text{M}$, $\text{HC} = 2.2(\pm 0.3)$, $p_{\max} = 0.62(\pm 0.03)$; cAMP: $K_{1/2} = 2,034(\pm 701) \mu\text{M}$, $\text{HC} = 1.5(\pm 0.1)$, $p_{\max} = 0.004(\pm 0.002)$; $n = 4$ (3 for $p_{\max}$)). RON-S2, N91–S240 of RET replaced by E89–R215 of OLF (cGMP: $K_{1/2} = 4(\pm 1) \mu\text{M}$, $\text{HC} = 3.0(\pm 0.3)$, $p_{\max} = 0.81(\pm 0.04)$; cAMP: $K_{1/2} = 1,135(\pm 300) \mu\text{M}$, $\text{HC} = 2.2(\pm 0.5)$, $p_{\max} = 0.7(\pm 0.1)$; $n = 6$ (3 for $p_{\max}$)).

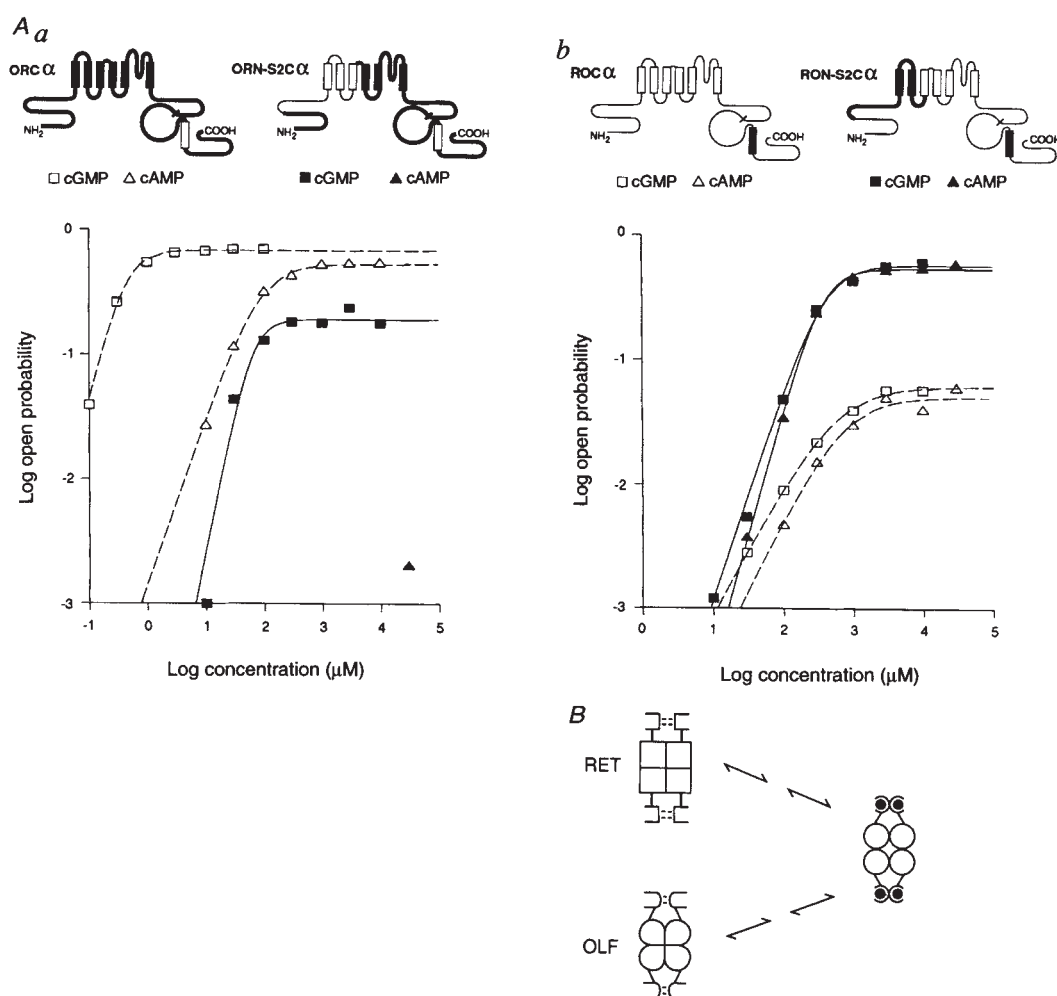
with the binding of cGMP to the retinal channel. The low efficacy with which cAMP and cGMP activate ROC can thus be explained by the coupling of a small free energy change from ligand binding (due to the binding domain of the olfactory channel) to a large free energy change required for gating (due to the N-S2 domain of the retinal channel). Conversely, the high efficacy of cAMP in gating ORC is explained because the small free energy change produced by cAMP binding to the retinal binding site is sufficient to activate the more readily opened olfactory N-S2 domain.

We used a simple allosteric model¹⁷ as a framework for interpreting these results. In the model, a tetrameric channel¹⁸ exists in either a closed (T) or open (R) conformation, whose equilibrium in the absence of agonist depends on the allosteric constant L_0 (Table 1). Agonists activate the channel because their affinity for the open state is greater than their affinity for the closed state. Under our assumption that the N-S2 domain determines the ease of channel opening (that is, L_0), the model can fit the dose-response curves for wild-type and C-terminal chimaeras (Fig. 1 and Table 1). Our interpretation is thus consistent with the data. The model also suggests why channels with a given

binding domain have a higher agonist affinity when coupled to the olfactory N-S2 domain than when coupled to the retinal N-S2 domain (that is, ORC versus the retinal channel and the olfactory channel versus ROC). Thus, allosteric coupling of channel opening to enhanced agonist binding implies that channels that open more readily (owing to the olfactory N-S2 domain) should also exhibit a higher agonist affinity than channels that are more difficult to open (because of the retinal N-S2 domain) (Fig. 4b).

What is the nature of the conformational change associated with channel opening? Recent experiments on subunit assembly of voltage-gated K^+ channels have identified a region in the N terminus before S1 (refs 19–21) and the S1 domain^{20,21} as sites of subunit tetramerization. Assuming that the homologous N-S2 domain is also a site of intersubunit contact in CNG channels, we propose that channel opening may involve a change in the strength of intersubunit bonds between N-S2 domains associated with an alteration in subunit orientation, similar to the allosteric conformational change in haemoglobin²² and gating of gap junction channels²³. A change in subunit orientation could increase agonist affinity through a change in C-helix–C-helix orientation

FIG. 4 Two domains are responsible for ligand activation. A, Exchanging both C-helix and N-S2 domains confers appropriate agonist-gating properties. a, OLF N-S2 domain and C-helix replaced by RET domains. Open symbols, representative dose-response curve for ORC_{α} (OLF with RET C-helix). Filled symbols, dose-response curve for the double chimaera $ORN-S2C_{\alpha}$ (OLF with RET N-S2 domain (RET S3 also included in this chimaera) and RET C-helix). cAMP only induced a measurable current when applied at 30 mM. b, Effect of replacing RET N-S2 domain and C-helix with corresponding regions from OLF. Open symbols, dose-response curve for ROC_{α} (RET with OLF C-helix). Filled symbols, dose-response curve for the double chimaera $RON-S2C_{\alpha}$ (RET with OLF N-S2 domain and C-helix). Squares: responses to cGMP; triangles: responses to cAMP. B, Allosteric model for channel activation. Closed RET channel is depicted by four squares, each representing a subunit in the closed state (T); strong interactions between subunits mediated by N-S2 domain (indicated by the large intersection between the subunits) stabilize RET in T state. Closed OLF is depicted as a cloverleaf with reduced interaction between the subunits. Open channels, indicated by circles, have even weaker subunit-subunit interactions. RET binding sites are depicted as hemi-squares in the T state (poor complementarity with agonist) or hemi-circles in the R state (good complementarity). OLF binding site is depicted as a hemi-oval in the T state; reflecting its intermediate complementarity to agonist. Possible interaction and reorientation upon activation of the binding domains along their C-helices, which form a site of dimerization in CAP¹⁴, indicated by the dotted lines in T state and closer apposition in the R state.



on the basis of the structure of the dimeric protein CAP¹⁴, whose C-helices are important sites of subunit interaction and form an intersubunit hydrogen bond with cAMP bound to the other subunit (Fig. 4b). Given the similar structure of voltage-gated and CNG channels, a role for altered subunit contacts in channel opening may be widespread. □

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METHODS. Double chimaeras generated by subcloning a fragment of one chimaera insert into another chimaeric construct using convenient restriction sites. $ORN-S2C_{\alpha}$, amino acids L87–V239 and Q560–A583 of OLF replaced by V89–Y265 and K590–N613 of RET (cGMP: $K_{1/2} = 39(\pm 17) \mu M$, $HC = 2.5(\pm 1.1)$, $p_{max} = 0.3(\pm 0.1)$; cAMP: $p_{max} = 0.004(\pm 0.003)$; $n = 3$). $RON-S2C_{\alpha}$, amino acids N91–S240 and K590–N613 of RET replaced by E89–R215 and Q560–A583 of OLF (cGMP: $K_{1/2} = 398(\pm 115) \mu M$, $HC = 1.7(\pm 0.1)$, $p_{max} = 0.62(\pm 0.02)$; cAMP: $K_{1/2} = 571(\pm 197) \mu M$, $HC = 1.8(\pm 0.3)$, $p_{max} = 0.64(\pm 0.03)$; $n = 4$ (3 for p_{max})).

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Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains

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THE Src-homology-3 (SH3) domains of the *Caenorhabditis elegans* protein SEM-5 and its human and *Drosophila* homologues, Grb2 and Drk (refs 1–4), bind proline-rich sequences found in the nucleotide-exchange factor Sos as part of their proposed function linking receptor tyrosine kinase activation to Ras activation^{5–7}. Here we report the crystal structure at 2.0 Å resolution of the carboxy-terminal SH3 domain from SEM-5 complexed to the mSos-derived amino-acid sequence PPPVPPRRR. The peptide is found to bind in an orientation ('minus') that is precisely opposite to that observed previously ('plus' orientation) in other SH3-peptide complexes^{8,9}. This novel ability of peptide-recognition proteins to recognize peptides in two distinct modes may play an important role in the signalling specificity of pathways involving SH3 domains. Comparison of this structure with other SH3 complexes reveals how a conserved binding face can be used to recognize peptides in different orientations, and why the Sos peptide binds in this particular orientation.

The Sos peptide binds the SEM-5 SH3 domain in a left-handed polypyrrolidine-II (PPII) helix conformation (Fig. 1), as seen in other SH3-peptide structures^{8,9}. Peptide residues sit snugly in a set of shallow hydrophobic binding pockets, referred

to as sites P₋₃, P₋₁, P₀, P₊₂ and P₊₃ as described previously⁸. There is a set of three hydrogen bonds between protein side chains and the peptide carbonyl oxygens.

Although this array of hydrogen bonds and binding pockets appears to be stereospecific, all interactions shown in Fig. 1c are also found in complexes of the opposite (plus) orientation (Fig. 1d), indicating that they are not a feature peculiar to either orientation. This network of interactions can recognize a PPII helix in both orientations because the helix is pseudo-symmetric (Fig. 2). In each orientation the proline rings occupy the same positions, and carbonyl groups are in positions that allow them to interact with the same hydrogen-bond donors. These interactions can therefore be used to recognize a basic PPII scaffold without specifying orientation or register.

Peptide residues Arg 7 and Val 4 are involved in specific, asymmetric interactions which appear to determine the orientation of the Sos peptide. The aliphatic portion of Arg 7 packs against the face of Trp 191, and the guanido group forms a charge stabilized hydrogen bond to Glu 172 (Fig. 3a). Glu 172 is in a protein segment called the RT loop¹⁰, which has a variable sequence in the SH3 family. Interactions between the residue at site P₋₃ and the RT loop seem to be important in determining binding orientation, as different non-proline residues also bind here in plus-oriented complexes (Fig. 1d)^{8,9}. A second asymmetric interaction which helps define binding orientation and specificity is that of Val 4 with the P₀ binding pocket (Fig. 3b). Using the repacking program PROPAK¹¹ we found that a proline at this position would pack less efficiently than a valine.

Yu *et al.*⁸ have argued that a preference for non-proline residues at particular surface-binding sites determines the specificity of the SH3 domain of phosphatidylinositol-3-OH kinase (PI(3)K), proposing that residues at sites P₀ and P₊₃ must be proline, with non-proline residues being tolerated or preferred at P₋₃, P₋₁ and P₊₂. Aligned peptide sequences that are thought to bind in the plus orientation satisfy these constraints (Table 1a). On the other hand, positional alignment of the Sos peptide, and of other peptides thought to bind in the minus orientation,

TABLE 1 Positional alignment of SH3 binding sequences shows correlation of required prolines with external packing sites

(a) Structure-based alignment of SH3 binding sequences

		Binding position										Origin	SH3 domain	Ref.
Orientation		P ₋₃	P ₋₂	P ₋₁	P ₀	P ₁	P ₂	P ₃						
Plus	N-	R	K	L	P	P	R	P	-C	RLP1*	PI-3K	8†		
	N-	R	A	L	P	P	L	P	-C	*	Src	8		
	N-	M	P	P	P	L	P	P	-C	3BP1	Abl	15‡		
	N-	Y	P	P	P	P	V	P	-C	3BP2	Fyn	15‡		
Minus	C-	R	P	P	V	P	P	P	-N	mSos1-1	Grb2/Sem5	6‡		
	C-	R	P	P	L	P	P	P	-N	mSos2-1	Grb2/Sem5	6		
	C-	R	P	P	L	L	P	P	-N	mSos1-2	Grb2/Sem5	6		
	C-	R	P	P	P	P	P	S	-N	mSos2-2	Grb2/Sem5	6		
	C-	R	P	P	V	P	P	G	-N	mSos1-3	Grb2/Sem5	6		
	C-	R	P	P	V	P	P	A	-N	mSos2-3	Grb2/Sem5	6		
	C-	R	R	P	L	P	P	H	-N	*	Src	8		
	C-	R	S	P	V	P	P	A	-N	Dynamin	PI-3K	16		
	C-	R		P	V	P	P		-N					

(b) Type of packing at central binding positions

	P ₋₁	P ₀	P ₁	P ₂	P ₃
Plus	In	Ex	—	In	Ex
Minus	Ex	In	—	Ex	In

Nomenclature for binding positions is from ref. 8 (Fig. 1). Positions boxed in the thin lines are those that contact the SH3 domain surface. Positions boxed in the heavy line are those that apparently require proline, in the respective orientations. Packing types are classified as either internal (In) or external (Ex), as described in Fig. 4 legend.

* Identified from screening of chemically synthesized peptide libraries.

† NMR structure of complex⁸.

‡ Crystal structures of complexes: Abl/3BP1 and Fyn/3BP2 (ref. 9); SEM-5/Sos, this work.

FIG. 1 Structure of the SEM-5/Sos peptide complex. *a*, An $F_o - F_c$ simulated annealing omit map¹⁷ of the Sos peptide electron density (1.5σ), superimposed on the final model (stereo) clearly defines the orientation and structure of the bound peptide. *b*, Sos peptide bound to the surface of the C-terminal SEM-5 SH3 domain. Surface is coloured by electrostatic potential, showing the negatively charged pocket (red) that binds Arg 7 of the peptide. Residues of the protein contacting the peptide are in green. Surface binding pockets⁸ are labelled in black. Peptide binding results in the burial $\sim 300 \text{ \AA}^2$ of solvent-accessible surface area. Hydrogen-bond distances (dotted lines) are: N α of Asn 56 to O of Pro 3, 3.3 \AA; OH of Tyr 57 to O of Pro 2, 2.7 \AA; and pyrrole N of Trp 42 to O of Pro 6, 3.0 \AA. *c*, Schematic diagram of binding pockets and hydrogen bonds observed in both plus and minus orientation complexes. The left-handed PPII helix, with 3 residues per turn, is depicted as a bent ribbon sitting on top of the SH3 domain binding surface. All labelled residues are highly conserved in the SH3 family. *d*, Identical views of the minus SEM-5/Sos complex and the plus Abl SH3/3BP1 complex⁹. Note the similarity in conformation of Arg 7 in the SEM-5 complex to that of Met 4 in the Abl complex, and the positioning of both residues near the variable RT loop. Generation of *a* was by the neon utility of the program MIDAS¹⁸; *b* by the program GRASP¹⁹, and *d* by the program RIBBONS²⁰.

METHODS. The C-terminal SEM-5 SH3 domain (residues 155–214) was purified as described²¹, except that the histidine tag was cleaved using cyanogen bromide instead of enterokinase. The complex was crystallized by vapour diffusion at 4 °C from 52.5% saturated NH_4PO_4 , 100 mM Tris, pH 8.0, 5 mM DTT mixed 1:1 with a solution containing protein (7.5 mg ml⁻¹) and a 3-fold molar excess of the peptide Ac-PPVPVPPRRR in 15 mM sodium acetate, pH 3.9. This peptide binds with a $K_D = 43 \mu\text{M}$ ²¹. Crystals grew to dimensions of $\sim 0.4 \times 0.4 \times 0.1$ mm and had 2/m symmetry. Upon mounting and initial diffraction characterization, unit cell dimensions shifted and stabilized at $a = 26.5 \text{ \AA}$, $b = 68.5 \text{ \AA}$, $c = 35.5 \text{ \AA}$, $\beta = 94.7^\circ$. Data to 2.0 \AA (completeness, 92%, $R_{\text{merge}} = 0.08$) were collected on a MacScience DIP2000 image plate detector, and processed using the programs DENZO and SCALEPACK (Z. Otwinowski, Yale University). Diffraction symmetry and systematic absences were consistent with space group $P2_1$. A crystal volume per unit molecular mass (V_m) of 2.0 \AA³ per dalton was consistent with two independent complexes per asymmetric unit. The structure was solved by molecular replacement using the program AMORE²². An all-alanine version of the spectrin SH3 domain²³, with 7 additional conserved hydrophobic side chains, was used as a search model. Two mutually consistent rotation–translation solutions, related by a local 2-fold axis, were subjected to positional and *B*-factor refinement using XPLOR²⁴. Density for many side chains not present in the search model was immediately visible. New side chains were built using the program XtalView (D. McRee, Scripps Research Institute), and refined using XPLOR. Density was clearly interpretable for residues 1–7 of the Sos peptide for each protein monomer. The structure shows that in the crystal two protein monomers are disulphide-linked via Cys 209. We postulate that disulphide bond formation was initiated by removal from solvent, depletion of DTT, and exposure to X-rays, and that formation of the linkage may have caused the shifts in unit cell dimensions. The final model includes 131 amino acids and 67 water molecules, and has a crystallographic *R*-factor of 0.189 (7–2.0 \AA) with root-mean-squared deviations in bond lengths and bond angles of 0.013 \AA and 1.8°, respectively. *B*-factors of the peptide atoms, particularly for those at the C terminus, are in general higher than those of the protein atoms, perhaps due to destabilization of the C-terminal salt bridge by the high ionic strength of the solvent. The visible arginine may adopt several conformations, although only the salt-bridged conformation appears to be significantly occupied. Several features of simulated annealing omit maps clearly indicate the orientation of the peptide: (1) distinctive density is observed for the non-proline residues Val 4 and Arg 7; (2) the density for the N-terminal Ac-Pro is very strong, and ends abruptly, indicating that this is the true start of the peptide; and (3) the detailed arrangement of protruding proline and carbonyl group density is more consistent with the minus orientation than the plus orientation. Even if Arg 7 is omitted from the model, refinement with a single peptide in the plus orientation results in a R_{free} (ref. 25) value that is 0.5% higher than with the peptide in the minus orientation.

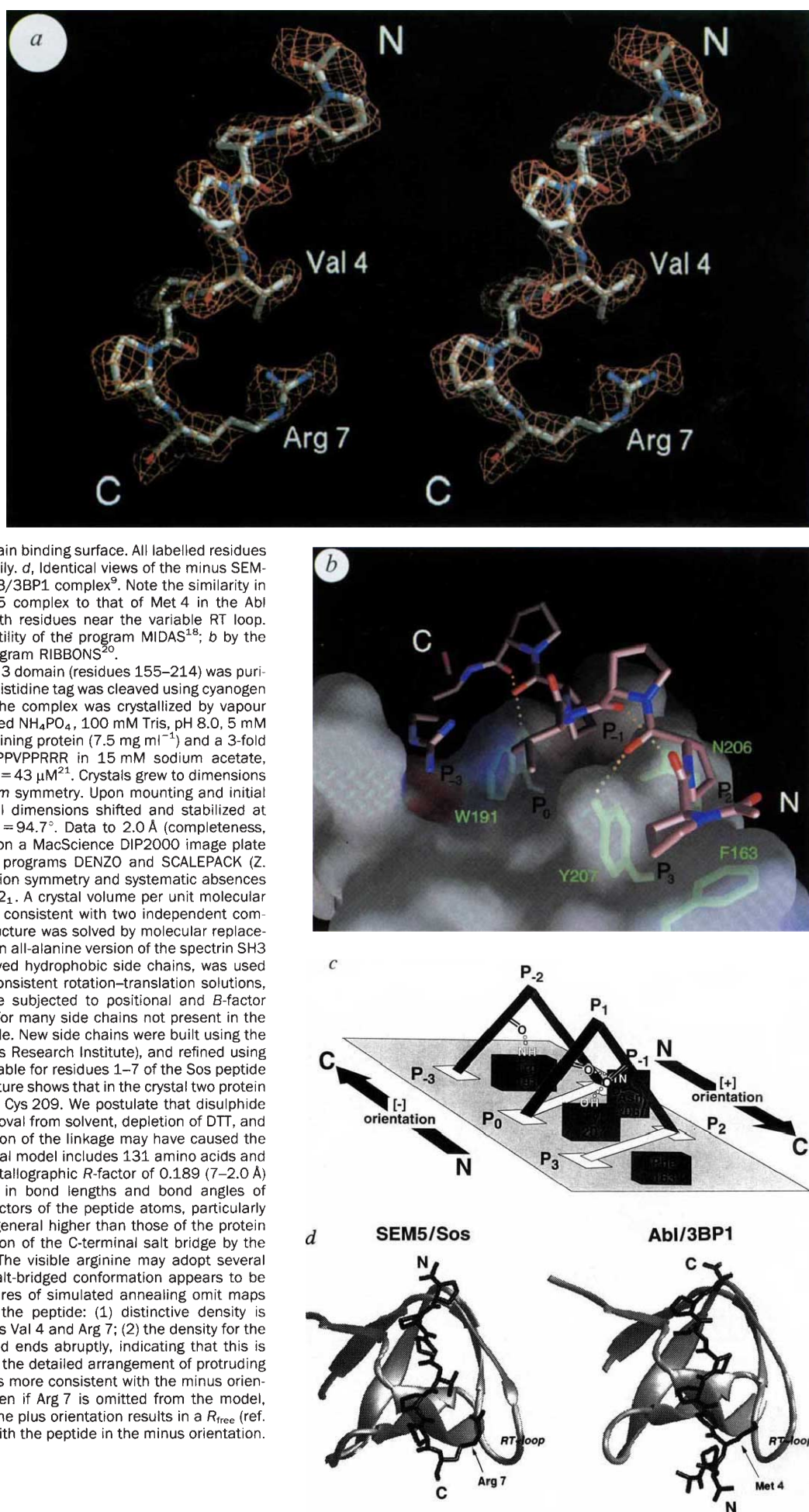


FIG. 2 Pseudo-symmetry of a PPII helix allows it to participate in identical packing and hydrogen-bonding interactions in both orientations. *a*, PPII helical wheel, viewed down the 3-fold helical axis, showing one turn of an ideal all-proline helix in two orientations. The helix on the left has the C terminus coming out of the page; that on the right has the N terminus coming out of the page. Side views are shown underneath. Both sets of proline rings can pack into the surface binding pockets of an SH3 domain, with pseudosymmetrically related side chains labelled A, B, C and A', B', C', respectively. *b*, Carbonyl C=O vectors of a PPII helix are positioned so that they can interact with the same protein hydrogen-bond donors in either binding orientation. The aligned, oppositely oriented helices are shown as fine lines, and C=O vectors as thick arrows. The set of shaded vectors are from the peptide oriented with the C terminus coming out of the page, and the set of black vectors are from the peptide oriented with the N terminus coming out of the page. A side view of the aligned helices is shown at the bottom.

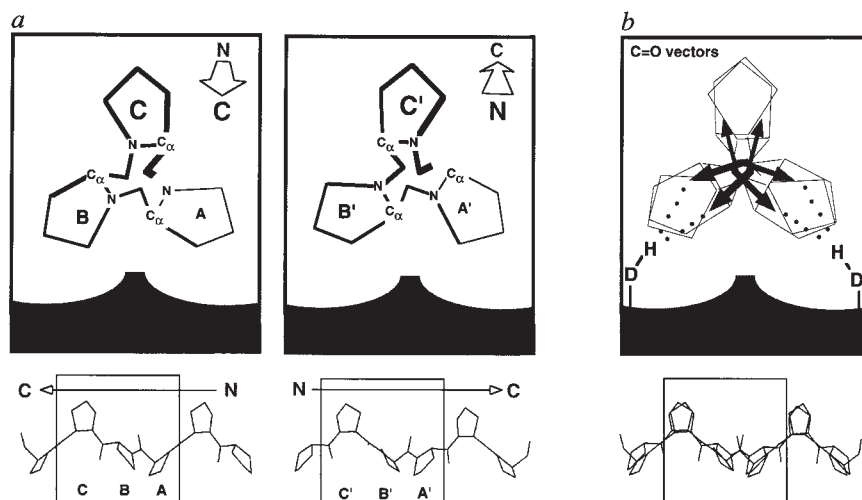
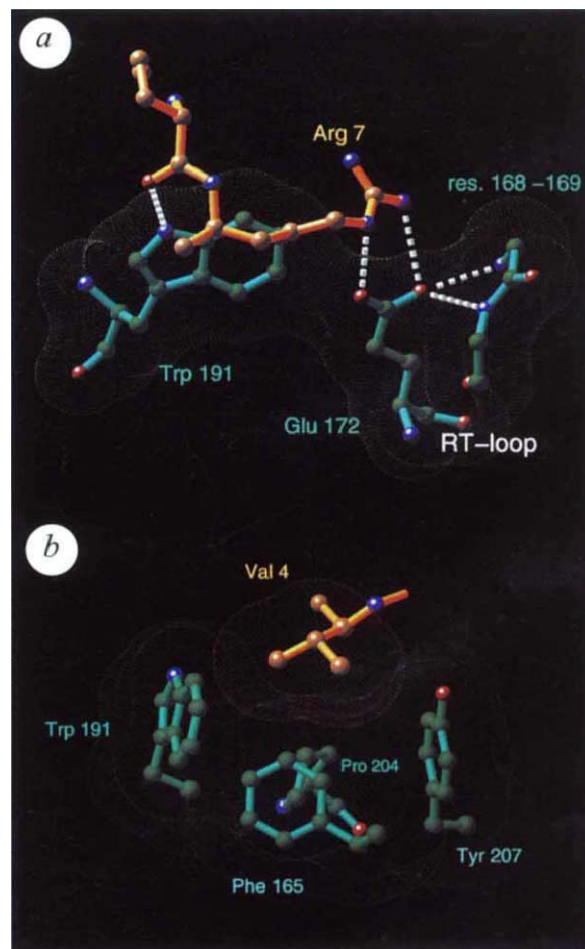


FIG. 3 Asymmetric interactions in the SEM-5/Sos complex that help define binding orientation. *a*, Peptide residue Arg 7 (orange), which binds at the P_{-3} site, shown with interacting protein residues (green). The aliphatic portion of the side chain packs against the side chain of Trp 191; the guanidino group forms a charge-stabilized hydrogen bond with protein residue Glu 172. In turn, Glu 172 is held in position by hydrogen bonds to the main-chain amide nitrogens of residues 168 and 169. Mutational studies corroborate the importance of this salt bridge, maintenance of a negative charge at position 172 being crucial for binding²⁶. These residues are part of the RT loop which varies in sequence within the SH3 family. The peptide residue preceding Arg 7 is hydrogen-bonded by its main-chain carbonyl to the pyrrole nitrogen of protein residue Trp 191. *b*, Peptide residue Val 4 (orange) makes favourable stereo-specific packing interactions with binding pocket P_0 . The pocket is defined by protein residues Phe 165, Trp 191, Pro 204 and Tyr 207 (green). Molecular surfaces are shown for the contacting protein residues. Using the program PROPAK¹¹, we have calculated that replacing Val 4 with proline would refill less than 75% of the volume originally occupied by the valine.



reveals that non-proline residues are found only at P_{-3} , P_0 and P_{+3} . Thus, although the binding pockets in these interactions are defined by a set of highly conserved residues, tolerance of non-proline residues at the homologous sites is reversed according to peptide orientation.

We believe this reversal in specificity occurs because, although a PPII helix is pseudo-symmetric, the atomic details of packing against the SH3 surface are different in the two orientations (Fig. 4). In one case the residue packs in a pocket with the α -carbon oriented away from the protein surface relative to the neighbouring main-chain nitrogen; in the other, the residue packs with the $C\alpha$ oriented towards the protein surface (defined as external and internal packing, respectively). It is because the $C\alpha$ and N atoms in the proline ring are almost structurally equivalent that a proline residue can interact similarly in both modes of packing.

If a non-proline residue is introduced in the helix, the $C\alpha$ and backbone N are no longer structurally equivalent. Non-alanine side chains almost universally adopt the favourable χ_1 torsion angles of approximately -60° or 180° (refs 11, 12). Thus at an internal packing site, a non-proline side chain would be oriented into the binding pocket, allowing it to make the same, or maybe better, van der Waals and hydrophobic interactions as a proline (Fig. 4a). But at an external packing site, a non-proline side

chain will be oriented away from the protein surface and will be unable to fill the binding pocket. Introduction of methylene-sized packing defects into folded proteins result in a 1.0–3.0 kcal mol⁻¹ loss in stability¹³, a decrease in energy that would greatly impair binding by SH3 domains. Internal and external packing modes differ in the relative position of the N and C α atoms. Therefore, at an external site, only proline, with its *N*-alkyl substitution, can mimic the packing interactions made by a non-proline side chain at an internal site. Thus prolines would be strongly favoured at positions with external packing.

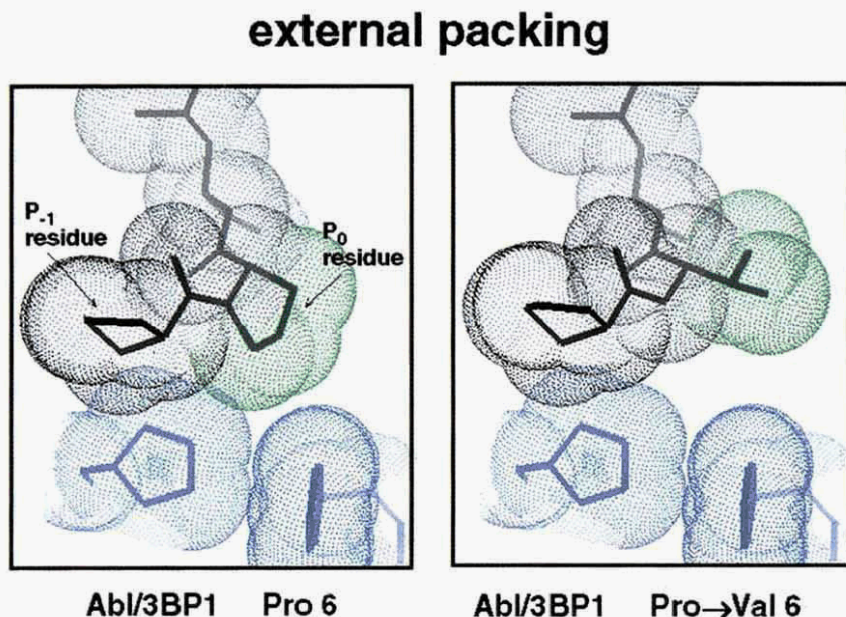
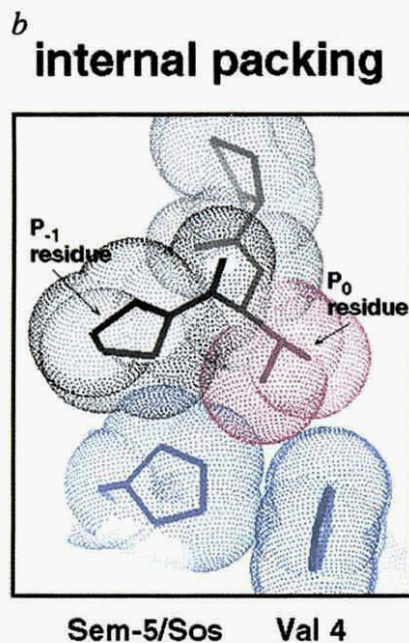
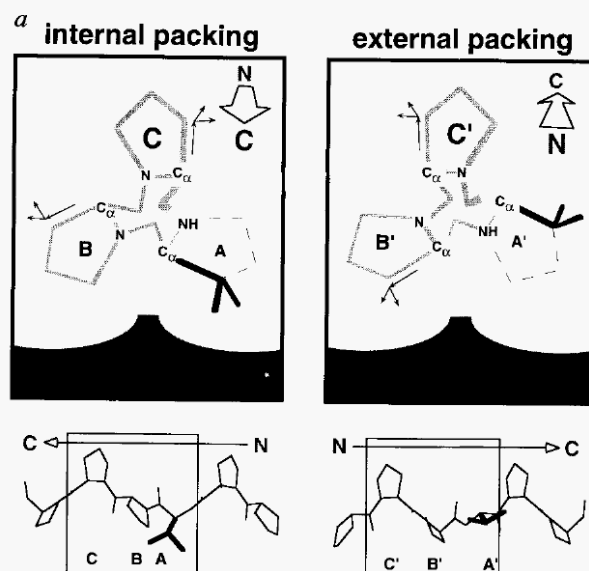
In both orientations, the central four binding pockets alternate between external and internal packing. More importantly, packing patterns are reversed for the two orientations (Table 1b). In the plus-orientated structure, the sites that require proline, P₀

and P₃, have external packing, whereas sites favouring non-proline residues, P₋₁ and P₂, have internal packing. The specificity is reversed, as expected, for the minus orientation, in accordance with the reverse packing pattern. Thus an SH3 domain's sequence preference within the proline-rich core is a function of peptide-binding orientation.

In summary, we propose that three factors interdependently determine the polarity and specificity of peptide binding to an SH3 domain. (1) A proline-rich core, which favours formation of the PPII helix scaffold, is necessary for the peptide to achieve general steric and hydrogen-bonding complementarity with the conserved central binding face. These interactions, however, do not define peptide orientation or register. (2) Interactions of residues outside the proline-rich core with non-conserved resi-

FIG. 4 Two types of packing geometries, with different preferences for proline residues, are observed at SH3 binding pockets. *a*, PPII helical wheels in two orientations as in Fig. 2, showing the differential effect of inserting a non-proline residue at positions A or A', which interact with the same binding pocket. Corresponding side views are shown directly underneath. Valine (bold) is used as an example of a non-proline residue. Dashed lines indicate the position that would be occupied by a proline. Residue A exhibits internal packing, the C α being displaced towards the protein surface relative to its neighbouring main-chain atoms. In this geometry, a non-proline residue, inserted in favourable rotamer conformations ($\chi_1 = -60^\circ$ or 180°), will be oriented into the binding pocket and will be able to achieve equal or better packing than a proline. Residue A' exhibits external packing, its C α being displaced away from the protein surface relative to its neighbouring main-chain atoms. In this geometry, a non-proline residue inserted in favourable rotamer conformations will be oriented away from the binding pocket and will not be able to pack into the pocket as well as a proline without introducing severe torsional strain or forcing large main-chain rearrangements which would disrupt the PPII helix. Prolines therefore appear to be required at external packing sites because the *N*-alkyl linkage of the pyrrole ring is the only available mechanism for placing side-chain bulk into the binding pocket. Fine arrows show the directionality of non-proline side chains if inserted at remaining positions. It is clear that for a PPII helix sitting on a surface, as in the SH3 interaction, residues on one side of the helix will show internal packing and residues on the opposite side will show external packing. *b*, Packing experimentally observed at site P₀ in the minus orientation SEM-5–Sos complex (internal packing), and the plus orientation Abl–3BP1 complex (external packing). Protein residues defining the base of the P₀ pocket (Phe 165 and Pro 204, in SEM-5) are shown in blue, the peptide residue binding

at the site in magenta or green, and remaining peptide residues in black. Val 4 in the SEM-5–Sos complex (left), and Pro 7 in the Abl–3BP1 complex (centre panel) both pack efficiently into the P₀ site, despite being in different packing geometries. A non-proline residue, however, such as valine, when modelled at the external packing Abl–3BP1 complex P₀ site in a favourable rotamer (right), leaves a large packing defect in the pocket.



dues in the SH3 domain, such as those in the RT loop, help to specify peptide orientation and register. (3) In conjunction with these interactions, packing geometry dictates what pattern of non-proline residues within the proline-rich core is compatible with the two different orientations. In the plus orientation, proline residues appear to be required at the P₀ and P₃ sites, and non-proline residues are allowed/favoured at the P₋₁ and P₂ sites, with converse requirements for the minus orientation. Satisfaction of all these three criteria seem to be necessary for a peptide to function as a high-affinity ligand.

The diversity of SH3 binding modes reported for various structures shows that SH3 domains are distinct from other families of peptide-recognition proteins such as antibodies, major histocompatibility complex molecules, or peptidases, which bind peptides in a fixed orientation¹⁴. SH3 domains, in contrast,

appear to be flexible units which, in conjunction with their ligands, can be evolutionarily tuned for a range of sequence and/or orientation specificities to meet the need for a particular recognition event. As these domains often link together components of a molecular complex, their functional specificity in the cell may not be determined by the full range of sequences that they can bind, but rather by the subset of sequences that they bind in the orientation required for the assembly of an active signalling complex. Correct geometry of complex assembly may be particularly important in interactions that involve the cooperative binding of proteins with multiple SH3 domains, such as SEM-5, to multivalent targets.

Note added in proof: NMR studies by M. Wittekind *et al.*²⁷ and H. Terasawa *et al.*²⁸ have shown that the N-terminal SH3 domain of Grb2 also binds Sos-derived peptides in the minus orientation. □

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Crystal structure of the complex of rat neonatal Fc receptor with Fc

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THE neonatal Fc receptor (FcRn) transports maternal immunoglobulin G (IgG) to the bloodstream of the newborn. FcRn is structurally similar to class I major histocompatibility complex (MHC) molecules^{1,2}, despite differences in the ligands they bind (the Fc portion of IgG and antigenic peptides, respectively). A low-resolution crystal structure of the complex between FcRn and Fc localizes the binding site for Fc to the side of FcRn, distinct from the tops of the $\alpha 1$ and $\alpha 2$ domains which serve as the peptide and T-cell receptor binding sites in class I molecules. FcRn binds to Fc at the interface between the Fc C_{H2} and C_{H3} domains, which contains several histidine residues that could account for the sharply pH-dependent FcRn/IgG interaction³. A dimer of FcRn heterodimers observed in the co-crystals and in the crystals of FcRn alone² could be involved in binding Fc, correlating with the 2:1 binding stoichiometry between FcRn and IgG (ref. 4) and suggesting an unusual orientation of FcRn on the membrane.

Crystals of a 2:1 complex between soluble rat FcRn⁵ and the Fc fragment of rat IgG diffract anisotropically to between 4.5 and 6 Å (ref. 4). The structure was solved at 6.5 Å resolution by two methods: molecular replacement using the atomic resolution

structures of rat FcRn² and human Fc⁶, and multiple isomorphous replacement (Fig. 1a, b; Table 1). The limited resolution prohibits detailed analysis but permits localization of the interacting site of each of the proteins, which can be analysed using the higher-resolution structures of the individual molecules. There is no electron density in the isomorphous replacement map (Fig. 1a) or in difference Fouriers calculated with molecular replacement phases for the portion of the C_{H2} domains distal to the C_{H3} domains, suggesting disorder of this region of Fc. However, electron density is present for the region where Fc and FcRn interact, and the correct orientation of the C_{H3} domains was verified by a real space search and location of a heavy-atom site. The C_{H2} domains were placed in the same relative orientation with respect to the C_{H3} domains as is found in Fc structures^{6,7}, in agreement with the observed density.

FcRn and Fc residues near the binding interface (Fig. 1c) are listed in Table 2. A major contact area on FcRn involves the side of the $\alpha 1$ and $\alpha 2$ domains. The N-terminal portion of the FcRn light chain (β_2 -microglobulin; $\beta 2m$) is also involved in contacts to Fc, as suggested from the observation that monoclonal antibodies directed against rat $\beta 2m$ inhibit the pH-dependent FcRn/Fc interaction⁸. Fc contacts FcRn mainly at the junction between the C_{H2} and C_{H3} domains, overlapping with the Fc binding site for fragment B of protein A (ref. 6), as predicted from inhibition studies⁸. The presence of three to four histidines on Fc at the FcRn interface may be relevant to the suggestion⁹ that titration of histidines could account for the binding of IgG to FcRn at pH 6.5 and its release at pH 7.5 (ref. 3).

Two FcRn molecules bind to a single Fc both in solution and in the co-crystals⁴, but the packing in the crystal creates two distinct 2:1 FcRn/Fc complexes. In the first 2:1 complex, a dimer of FcRn heterodimers contacts a single Fc molecule asymmetrically, with the majority of the contacts to Fc involving one of the receptors (Fig. 2a). The same dimer of FcRn molecules is also observed as a non-crystallographic dimer in three crystal

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forms of FcRn alone². If this form of 2:1 complex exists on the cell surface, FcRn is predicted to be arranged with its long axis nearly parallel to the membrane ('lying down'). Contacts between Fc and the second FcRn molecule of the dimer are probable, involving the region around Glu 272 and His 285 on Fc and the region on FcRn near Leu 219 and Lys 245. This interaction is less certain than the main set of interactions owing to the absence of electron density for part of the C_H2 domain, but correlates with the observation that FcRn residues 219–224 affect IgG-binding affinity slightly⁸. The second 2:1 complex is a symmetrical arrangement of two FcRn molecules surrounding a single Fc (Fig. 2b) using the binding sites on both chains of the homodimeric Fc. In this complex, the FcRn molecules would be 'standing up' in the standard orientation assumed for MHC

molecules on the surface of a cell, with each FcRn making contacts only to Fc.

The presence of two distinct 2:1 FcRn/Fc complexes in the co-crystals raises the question of which is relevant to the FcRn/Fc complex on a membrane. Two lines of reasoning suggest the dimer of FcRn heterodimers in the 'lying down' 2:1 complex (Fig. 2a) is functionally relevant for Fc binding. First, rationalization of site-directed mutagenesis results⁸ requires the formation of the FcRn dimer of heterodimers. Alteration of His 250 and His 251, residues at the dimer interface², result in a receptor with reduced affinity for IgG (ref. 8), implying that the FcRn dimer of heterodimers² (Fig. 2a) is relevant for ligand binding. In addition, alteration of residues within the FcRn counterpart of the class I CD8 binding loop¹⁰ (α 3 domain residues 219 to 224)

TABLE 1 Data collection statistics

X-ray source/No. of crystals	Derivative/ Unique*/ Redundancy§	Resolution (Å)/ R_{merge} (%)†/ Complete (%)	Resolution (Å)/ R_{merge} (%)†/ Complete (%)	MFID (%)¶	R.m.s. $f_h/E^\#$	Mean FOM‡ (25–6.5 Å)¶
CHESS* merged with rot. anode	Native** 8,524	25–4.5 12.5	4.7–4.5 28.9			0.35
2	3.0	67 (94% to 7 Å)	23			
Rotating anode	HgCl ₂ 3,019	25–6.5 9.6	6.8–6.5 31.6	30.4	0.8	
1	2.8	69	66			
Rotating anode	K ₂ PtCl ₄ 3,397	25–6.5 9.2	6.8–6.5 27.3	26.1	1.0	
1	2.5	80	43			
CHESS*	K ₂ PtCl ₄ 2,556	25–6.0 9.5	6.3–6.0 27.3	35.0	1.2	
1	1.7	54	45			

Crystallization and data collection. A 2:1 complex between soluble rat FcRn⁵ and rat Fc (a mixture of the four rat IgG subtypes; Jackson Immunoresearch Labs) was isolated and crystallized as described⁴. Despite cryopreservation and data collection using high-intensity synchrotron radiation, the crystals (space group $I2_12_12_1$, $a=125$ Å, $b=145$ Å, $c=216.5$ Å, 80% solvent, 1 FcRn plus $\frac{1}{2}$ Fc per asymmetric unit) diffract to only 6 Å, with anisotropic diffraction to 4.5 Å in the a/a^* direction. Attempts to improve the quality of the crystals by crystallizing a complex containing a monoclonal rat Fc fragment or Fc fragments of other species were unsuccessful. Crystals were stored in 0.1 M citrate, pH 5.6, 0.67 M sodium tartrate, 0.2 M MgCl₂ (handling buffer). Before low-temperature data collection, crystals were transferred to handling buffer containing 20% ethylene glycol in stepwise increments, mounted in glass loops¹³, and cooled to -170 °C in a nitrogen gas stream using a Molecular Structure Corporation low-temperature device. Data were collected on an R-Axis IIC X-ray detector mounted on a Rigaku rotating anode or on Fuji HR-III images plates at the Cornell High Energy Synchrotron Source (CHESS). Data were integrated using DENZO (Z. Otwinowski, personal communication) and further processed using CCP4 software¹⁴. **Molecular replacement.** The non-crystallographic FcRn dimer of heterodimers² was used as a search model to increase the sensitivity of the search, because the model describes the Patterson vectors across the dimer interface as well as the vectors within a single FcRn molecule. The highest peaks obtained from the Patterson space rotation search gave the best correlation after Patterson-correlation refinement¹⁵ (correlation 0.11, average 0.04, $\sigma=0.01$, 10–5 Å). The FcRn dimer was then used as a search object in the three-dimensional translation function programme within X-PLOR¹⁶. A solution of 10σ (the next highest peak was 1.5σ lower) was obtained for spacegroup $I2_12_12_1$, aligning the FcRn dimer along a crystallographic two-fold axis. No clear solution was found for space group $I222$. A six-dimensional real-space search² was used to place a single C_H3 domain of Fc (Entry 1FC2 in the Protein Data Bank) in a $2F_o - F_c$ map calculated with phases derived from the molecular replacement solution of the FcRn dimer, resulting in a correlation between map and model of 10.5σ higher than the average correlation for a random placement. A crystallographic two-fold axis generated the partner C_H3 domain in its correct orientation (so that the two domains were paired with the orientation observed in the Fc structure⁶), validating the correctness of the real-space search solution. Although clear electron density was not seen for the entire C_H2 domain, inclusion of the C_H2 domain (including the carbohydrate) lowered the R -factor slightly (see below). The final rotation and translation values for the C_H2 domain correspond to a clear minimum in the R -factor. After rigid body refinement using five domains (Fc C_H2 and C_H3, FcRn $\alpha 1\alpha 2$, $\alpha 3$ and $\beta 2m$), refinement of overall B -factors for the domains, and refinement of the overall anisotropic B -factor, the R -factor was 42.3% for all reflections between 20 and 4.5 Å (43.4% when the C_H2 domain was excluded). The relative orientation of the C_H2 domain with respect to the C_H3 domain stayed close (10–16° different) to that observed in the structures of Fc⁶ and of an intact IgG⁷ during refinement, but differed more from the orientation in the structure of Mcg IgG1 (ref. 17), which could be due to a hinge deletion in that antibody. **Isomorphous replacement.** Crystals were soaked overnight in 3 mM HgCl₂ plus 3 mM *N*-acetylhomocysteine thiolactone as described² or in 2 mM K₂PtCl₄. Molecular replacement phases were used to locate the heavy-atom positions. Heavy-atom sites on FcRn were the same as observed previously², with an additional platinum site on Met 358 of the C_H3 domain of Fc. Heavy-atom refinement and phasing was done with MLPHARE¹⁸ and isomorphous replacement maps were improved using solvent flattening implemented in reciprocal space¹⁹.

* Unique, number of unique reflections.

† R_{merge} (%) = $100 \times (\sum |I - \langle I \rangle| / \sum I)$, where I is the observed intensity and $\langle I \rangle$ is the average intensity from several measurements.

‡ FOM, figure of merit.

§ Redundancy, (number of measurements)/(number of independent reflections).

|| Complete (%) = $100 \times (\text{number of independent reflections}) / (\text{maximum number theoretical})$.

¶ MFID (mean fractional isomorphous difference) = $\sum |F_{\text{ph}} - F_p| / \sum F_p$, where F_p is the protein structure factor amplitude and F_{ph} is the heavy-atom derivative structure factor amplitude.

R.m.s. f_h/E (phasing power), where f_h is heavy-atom structure factor amplitude and E is residual lack of closure error.

* CHESS, Cornell High Energy Synchrotron Source.

** Only reflections with $1/\sigma > 1.0$ were used.

slightly lowered the affinity of interactions between FcRn and IgG (ref. 8). There are no predicted contacts between Fc and the FcRn $\alpha 3$ domain in the 'standing-up' 2:1 complex (Fig. 2b). However, in the lying-down orientation (Fig. 2a), Fc binding to the $\alpha 1$ and $\alpha 2$ domains of one FcRn may contact the appropriate region of the $\alpha 3$ domain of the dimer-related partner FcRn molecule (mutated residues are highlighted on the FcRn structures in Fig. 2a and b). Second, the Fab arms of an intact IgG could easily be accommodated on either side of the FcRn dimer in the lying-down 2:1 complex (Fig. 2a), but computer modelling suggests that intact IgG would be sterically prohibited from binding to cell-surface FcRn in the standing-up complex (Fig. 2b) owing to collisions with the membrane surface. These

two points argue for the involvement of the FcRn dimer of heterodimers in the binding of Fc. Perhaps relevant to the situation on a cell membrane is the fact that FcRn is a monomer (that is, a single heterodimer) in solution³, and a single FcRn molecule can bind Fc⁴. The C termini of the two FcRn molecules in the lying-down 2:1 FcRn/Fc complex are 32 Å apart, close enough to allow an interaction between the two cytoplasmic domains. An attractive feature of the lying-down model is that a signal for endocytosis of the FcRn/IgG complex could be binding of an IgG to a single FcRn, followed by recruitment of a second FcRn to form an FcRn dimer in which the cytoplasmic domains are spatially proximal. Mutagenesis of the FcRn dimer interface should allow testing

FIG. 1 The FcRn/Fc complex. *a*, Stereo view of the 6.5 Å solvent-flattened¹⁹ multiple isomorphous replacement map contoured at 1.1 σ . The carbohydrate of Fc and the α -carbon backbones of one FcRn and one Fc are shown in black lines. A portion of the backbone of a second FcRn molecule forming the FcRn dimer (Fig. 2a) is shown in red. The backbones of other symmetry-related molecules have been omitted for clarity. To fit the electron density map, the coordinates of FcRn² and human Fc⁶ (entry 1FC2 in the Protein Data Bank) were used without alteration and subjected to rigid body refinement. Electron density for the C_H2 domains of Fc is weak or missing outside the portion of the domains that are closest to C_H3. Portions of the C_H2 domains most distant from the C_H2-C_H3 interface in the human Fc and Fc/fragment B complex structures were also found to be poorly ordered or lacking electron density⁶. In the FcRn/Fc structure, the C_H2 domain electron density may be even weaker because rat Fc fragments (unlike human Fc fragments) are non-covalent (A.H.H., unpublished observations) owing to proteolytic cleavage C-terminal to the disulphide bonds that link the two IgG heavy chains in the hinge. Covalent and non-covalent forms of Fc bind to FcRn with the same pH dependence and approximately equal affinities²². *b*, Close-up stereo view of the 6.5 Å solvent-flattened¹⁹ multiple isomorphous replacement map contoured at 1.5 σ . The α -carbon backbone of the FcRn $\alpha 1$ and $\alpha 2$ domains² is shown as fit to the electron density, which is best in the helical regions of the map. The β -sheet secondary structure is not resolved at this resolution. These figures were generated with the program O (ref. 27). *c*, Stereo drawing generated with the program MOLSCRIPT²⁸ of the FcRn/Fc complex (one FcRn molecule, 1/2 of an Fc molecule) forming the asymmetric unit of the co-crystals (FcRn, thin line; Fc, thick line) in the same orientation as in *a*.

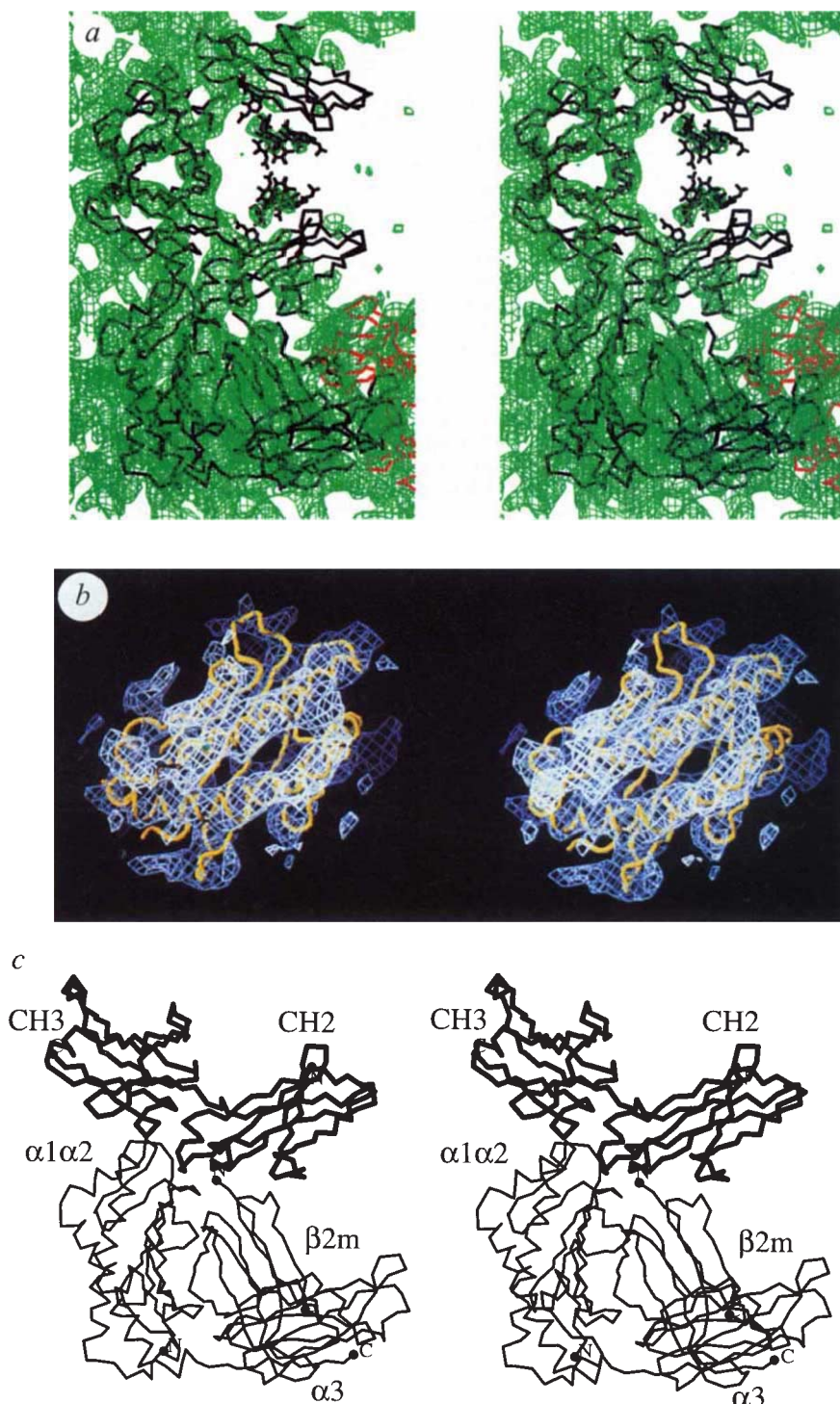


FIG. 2 *a, b*, The two different 2:1 FcRn/Fc complexes observed in the co-crystal. Each figure shows the contents of two asymmetric units. The α -carbon backbone of the FcRn heavy chain is shown in blue, β_2m in green and Fc in red; disulphide bonds are yellow. Residues identified by site-directed mutagenesis to affect the binding affinity of IgG (ref. 8) are highlighted (His 250 and His 251, magenta; residues 219–224, yellow). The plane of the membrane is assumed to be horizontal in each figure. *a*, The lying-down 2:1 FcRn/Fc complex. No steric hindrance between intact IgG and the membrane is expected in this orientation as the two Fab arms of IgG could project out of the plane of the paper (towards the viewer) and into the plane of the paper. Alteration of His 250 and His 251 (magenta) probably affects IgG binding affinity indirectly because of their location at the FcRn dimer interface² with no predicted contacts to Fc. Titration of these residues over from the permissive (pH 6.5 or lower) to non-permissive (pH 7.5 or higher) pH of IgG binding could partially explain the pH dependence of the FcRn/IgG interaction³. The effect of altering FcRn residues 219–224 (yellow) can be rationalized by the predicted contact between the Fc C_{H2} domain and residue 219 of the second FcRn molecule (Table 2b). *b*, Standing-up 2:1 FcRn/Fc complex. In this orientation, computer modelling (W.P.B. and P.J.B., unpublished results) suggests there is likely to be steric hindrance between intact IgG and the membrane, even when taking into account the flexibility of the Fab arms. (The Fab arms emanate from the hinge region N-terminal to the two C_{H2} domains, which would be close to the predicted location of the membrane. Accommodation of the two Fab arms would require an acute angle between the Fc and Fab arms, rather than the perpendicular or obtuse angles believed to be relevant for IgG structures^{7,17}.) Neither of the regions indicated by site-directed mutagenesis to affect IgG binding affinity⁸ are at the Fc interface or any other protein–protein interface in this model. *c*, Schematic view of the packing in the co-crystal to illustrate the possible network of 2:1 FcRn/Fc complexes forming on a membrane. The contents of four asymmetric units (each containing one FcRn molecule and one of the two polypeptide chains of homodimeric Fc) of the co-crystal are shown. Positions of crystallographic two-fold axes are indicated as arrows. The portion of the FcRn heavy chain following the C terminus of soluble FcRn and the transmembrane region (not in the structure of the co-crystals or of FcRn alone²) is modelled as an extended chain connecting the α_3 domain to the membrane. At the bottom of the figure, the two FcRn molecules bound to the left Fc molecule correspond to the 2:1 complex shown in *a*, and the FcRn molecules on the top and bottom left that surround a homodimeric Fc correspond to the 2:1 complex shown in *b*. Different shading indicates relative positions of the molecules in the third dimension. Existence of this network under physiological conditions would require two parallel membranes (indicated as thick black lines) separated by 160 to 170 Å. The separation between the membranes of adjacent microvilli in the brush borders of neonatal rat epithelial cells, where FcRn is expressed and functions, can be as little as 200 Å (ref. 11), raising the possibility that networks of 2:1 FcRn/IgG complexes could form between microvilli. Alternatively, this network of 2:1 complexes may exist only in the crystalline environment in which the protein concentration is very high. If the Fc portion of IgG is distorted when bound to an FcRn dimer of heterodimers, a second FcRn dimer might be prevented from binding to the other Fc polypeptide chain, preventing the formation of a network of complexes on the cell surface. Such a distortion of Fc could cause the C_{H2} domains in adjacent asymmetric units to adopt different conformations which could account for the weak electron density of the C_{H2} domains in the FcRn/Fc co-crystal. Even with a distorted Fc, the possibility of binding two Fc fragments to one FcRn dimer exists, but might be impossible when binding intact IgG because of steric hindrance between the Fab arms of the two molecules.

for the existence of the FcRn dimer in complex with IgG in solution or on the membrane.

The observation of a network of complexes within the FcRn/Fc co-crystal raises the possibility that both 2:1 complexes could exist on the cell surface. The formation of a network of complexes would require the presence of two parallel membranes (Fig. 2c), a situation that exists in the microvilli of brush borders of intestinal epithelial cells, where FcRn is expressed and

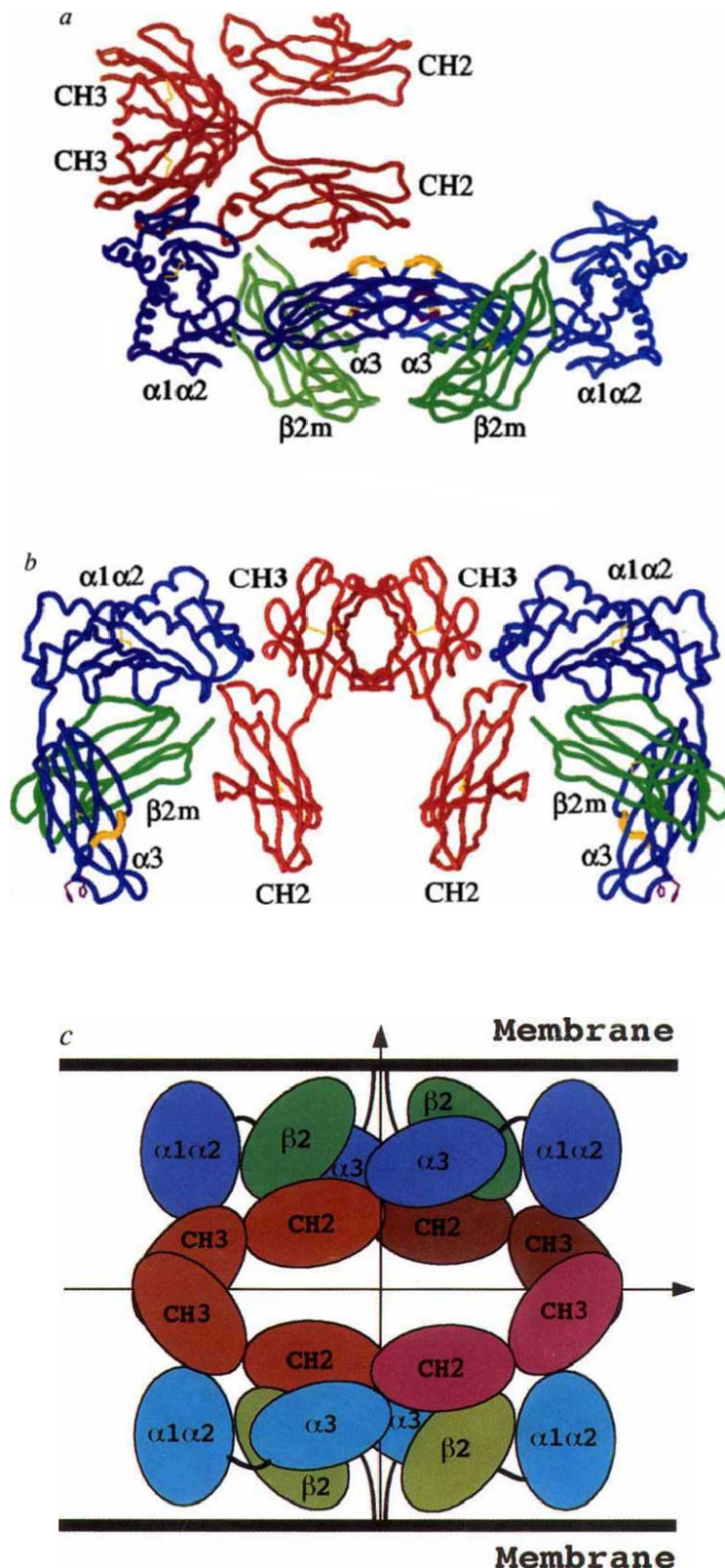


TABLE 2 Residues on Fc and FcRn at the predicted FcRn/Fc interface from low-resolution co-crystal structure

(a) Fc residues that potentially contact FcRn													(b) FcRn residues that potentially contact Fc				
Residue	Rat				Murine				Human				Residue	Rat	Murine		Human
	1	2a	2b	2c	1	2a	2b	3	1	2	3	4					
248	K	K	K	K	K	K	K	K	K	K	K	K	<i>α1 domain</i>				
250	V	V	I	L	V/T	V	V	A	T	T	T	T	84	N	K		G
251	L	L	L	L	L	L	L	L	L	L	L	L	85	Q	I		—
252	T	T	L	M	T	M	M	M	M	M	M	M	86	I	L		—
253	I	I	I	I	I	I	I	I	I	I	I	I	90	F	Y		Y
254	T	T	S	T	T	S	S	S	S	S	S	S	<i>α2 domain</i>				
255	L	L	Q	L	L	L	L	L	R	R	R	R	113	A	A		A
256	T	T	N	T	T	S	T	T	T	T	T	T	114	L	L		L
257	P	P	A	P	P	P	P	P	P	P	P	P	115	N	N		N
272*	E	E	D	D	E	D	D	D	E/Q	E	E	E	116	G	G		G
285*	H	H	H	F	H	H	H	H	H	H	H	H	117	E	E		E
288	Q	Q	Q	Q	Q	Q	Q	Q	K	K	K	K	118	E	E		E
290	R	H	Q	Q	Q	Q	Q	Q	K	K	K	K	119	F	F		F
291	P	A	P	P	P	T	T	P	P	P	P	P	131	G	G		G
308	I	I	I	I	I	I	I	I	V	V	V	V	132	E	E		D
309	L	V	Q	Q	M	Q	Q	Q	L	V	L	L	133	W	W		W
310	H	H	H	H	H	H	H	H	H	H	H	H	134	P	P		P
311	Q	R	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	135	E	E		E
314	L	L	M	M	L	M	M	M	L	L	L	L	136	T	T		A
385	G	G	G	G	G	G	G	G	G	G	G	G	137	D	E		L
386	Q	Q	H	E	Q	K/R	H	E	Q	Q	Q	Q	<i>α3 domain (of second FcRn molecule in lying-down model)</i>				
387	P	P	I	L	P	T	L	T	P	P	P	P	219*	L	L		L
428	L	L	V	V	L	V	R	V	M	M	M	M	245*	K	K		K
433	H	H	H	H	H	H	K	H	H	H	H	H	<i>β2m domain</i>				
434	N	N	N	N	N	N	N	N	N	N	N	N	1	I	I		I
435	H	H	H	H	H	H	Y	H	H	H	R	H	2	Q	Q		Q
436	H	H	H	H	H	H/L	Y	H	Y	Y	F/Y	Y	3	K	K		K
													4	T	T		T
													86	T	S		T
													88	K	A		S

Predicted interface residues that are nearly completely conserved, or in which structural features of the residues are conserved, are printed in bold. Residues involved in predicted contacts between Fc and the second FcRn in the FcRn dimer (Fig. 2a) are indicated by an asterisk. The criterion for inclusion in this list is a distance of less than 5 Å between an atom within the residue and any atom on the partner molecule. This table is intended as a rough map of the FcRn/Fc interaction rather than an exact atomic analysis, because of the low resolution of the co-crystal structure. Differences between human Fc and rat Fc would not be resolved at this resolution, nor would potential local changes in the structures of either molecule caused by binding. a, Fc residues that potentially contact FcRn. The residues for the four subtypes of human, rat and murine Fc are listed²⁰. All subtypes bind to rat FcRn with the same pH dependence²¹. Polyclonal rat and human IgG bind with roughly equal affinities to rat FcRn²². The order of affinities of binding within each species is: IgG2a > IgG1 > IgG2b = IgG2c (rat²³), IgG1 > IgG2b > IgG2a > IgG3 (murine²⁴), and IgG1 = IgG2 > IgG3 > IgG4 (human²⁵). b, FcRn residues that potentially contact Fc. The residues for rat¹, mouse²⁵ and human²⁶ FcRn are listed.

functions¹¹. Alternatively, networks of 2:1 FcRn/Fc complexes may not form on the membrane or in solution owing to a distortion of Fc that prevents one of the Fc polypeptide chains from binding FcRn, thereby disrupting the interaction that forms the standing-up complex (Fig. 2b). Distortion has been observed for the Fc portion of IgE, which is bent when bound to the IgE Fc receptor (FcεRI)¹².

Which of the 2:1 FcRn/Fc complexes in the co-crystal is relevant to the physiological situation on the cell surface is not yet known, but the co-crystal structure clearly shows that FcRn interacts with the Fc portion of IgG in a way distinct from the interactions of MHC molecules with peptides or macromolecular ligands. The structure of the FcRn/Fc complex adds another example to the variety of sites that are involved in recognition processes on proteins with the MHC structural motif. □

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Time to abandon Brussels' bid on patents

Gary Moss and Simon Cohen

After six years of debate, the European Commission's attempts to harmonize biotechnology legislation still face opposition over the patenting of genes. Recent legal experience suggests that its efforts are unnecessary.

NEXT week, a critical meeting takes place in Brussels which could well determine the fate of the European commission's six-year attempt to harmonize legislation on biotechnology patents among the 12 — soon to be expanded — members of the European Union (EU).

The meeting is of the conciliation committee, a body established under the Maastricht Treaty in order to resolve differences between the European Parliament and the Council of Ministers. The main difference to be resolved in this instance relates to the extent to which individual genes can be patented. As such, it goes to the heart of the future of the biotechnology industry.

Article 2 of the draft directive specifies three particular categories of inventions which are unpatentable: the human body or "parts of the human body as such"; processes for modifying the genetic identity of the human body "contrary to the dignity of man"; and processes for modifying the genetic identity of animals which are likely to cause them suffering or physical handicaps "without any substantial benefit to man or animal", and animals resulting from such processes.

The phrase "parts of the human body as such" is intended to mean parts of the human body as they are found inside the body. According to language elsewhere in the directive, this reaffirms the general principle that no-one should, by obtaining a patent, be able to claim ownership of, for example, a gene, protein or cell in its natural state in the human body.

In May, however, the European Parliament passed a proposed amendment to this section which would also bar patents on genes, proteins or cells that have been isolated from the body. The Parliament wants an explicit and unambiguous statement that no part of the human body can be patented — whether or not it has been isolated. In addition, it has proposed an absolute ban on patenting human genes and gene therapies.

The European Parliament is apparently concerned that the term 'as such' may be ambiguous, and might permit the type of patent application for gene sequences made (but subsequently withdrawn) by

both the US National Institutes of Health and Britain's Medical Research Council for sequences of genes whose functions had not been determined.

But the effect of the proposed amendment would be devastating. In particular, it would preclude patents on many types of therapeutic proteins which have only become available as a result of the efforts of scientists who have successfully cloned the relevant gene and expressed the protein in a suitable host.

If approved, the Parliament's proposal could cause major damage to the biotechnology industry. For it would remove the incentive to the pharmaceutical companies which support these scientists to continue to back their search for effective therapeutic treatments for the many gene-based illnesses. Indeed, the Parliament's recommendation raises the question of whether we need the directive at all. We would argue that we do not.

Current patent law across Europe conforms with the European Patent Convention (EPC), a pan-European agreement drawn up in 1973 and now signed by all member states of the European Union (in their individual capacities) as well as some non-EU countries (Austria, Sweden and Switzerland).

Inventors wishing patent protection in EPC countries can either apply to the European Patent Office (EPO) in Munich, designating the individual signatory states in which they want the patent protected, or make individual applications to national patent offices. The EPO route is usually preferred, as it involves a single application covering many states.

Many challenges have already been made to biotechnology patents which have led to hearings before the EPO's Opposition Division and Board of Appeal. In addition, both the EPO and national courts have handed down numerous judgments dealing with such patents.

Neither has felt the need for new legislation to deal with issues raised by genetic engineering, even though the technology has progressed enormously since the EPC was drawn up. This is primarily because the convention provides an adequate framework for courts to apply existing patent law to a particular case.

For an invention to be patentable, various hurdles need to be overcome. One is that it must involve an inventive step. The EPO usually evaluates this by deciding whether an invention solves a technical

problem in the field. In addition, the invention must also be industrially applicable. And the patent must not be "contrary to 'ordre public' or morality".

All three requirements already give patent offices and the courts the scope either to refuse applications, or to revoke patents such as those relating to gene sequences with no determined functions (as in the NIH applications), should they choose to do so.

The new directive, in the form now proposed by the commission, is not significantly different from the existing law as promulgated by the European Patent Offices and the national courts. As all EU member states are signatories to the EPC, it can be argued the laws of those states on this issue are already harmonized.

All the commission's efforts appear to have done is to provide a platform for lobby groups to mount a fierce attack on the whole principle of patenting the results of genetic research. If changes are required in the future, this could be done through an amendment to the EPC. There would be no need for the commission to get involved.

Moreover, European patent law is dynamic, constantly evolving to deal with new issues and developing technologies. Last month, for example, Britain's Court of Appeal set a controversial precedent when it handed down a judgment on a challenge from Medeva plc to Biogen Inc.'s patent on a hepatitis B vaccine produced by recombinant DNA technology.

Although the Appeal Court found against Biogen, its decision is widely seen as being out of line with established European patent law, and Biogen is likely to attempt to appeal the decision to the House of Lords. But whatever the outcome, no-one is suggesting that new legislation is required. The argument is over the interpretation of existing legislation.

It is far better to give courts the scope to rule on the desirability of biotechnology patents in this area under the existing provisions, rather than seek to place them in a straitjacket, with little room for manoeuvre.

The biotechnology directive in its current form is certainly unhelpful. Even more importantly, the directive is not needed. It should be shelved without further ado. □

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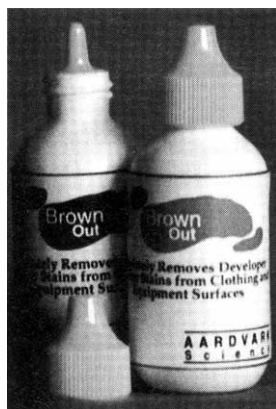
This is the second of an occasional series of articles on intellectual property rights and the law. The first article appeared in *Nature* 369, 589; 1994.

Molecular marketplace

Recent product announcements include atomic force microscopy for fluids, a protein research tool for molecular biologists and a system for positive-selection cloning of PCR products through blunt-end ligation.

DIGITAL Instruments has extended its Tapping-Mode **atomic force microscopy** technology to include operation in fluids (*Reader Service No. 100*). This special mode of operation can be used for non-destructive imaging of surfaces of soft materials and is said to provide sub-angstrom vertical resolution and nano-metre lateral resolution. Extending this technology to include fluids could be useful in biological applications where fragile samples are best imaged in native or near-native fluid environments or in preservation media such as alcohols or glutaraldehyde. This might include samples such as DNA, DNA-protein complexes, chromosomes, RNA transferase and whole cells.

Removing stubborn stains in clothing caused by the types of developers and fixers normally used in a scientific darkroom need not be a problem, says Aardvark Science, with its new **Brown-Out stain remover** (*Reader Service No. 101*). Brown-Out is not

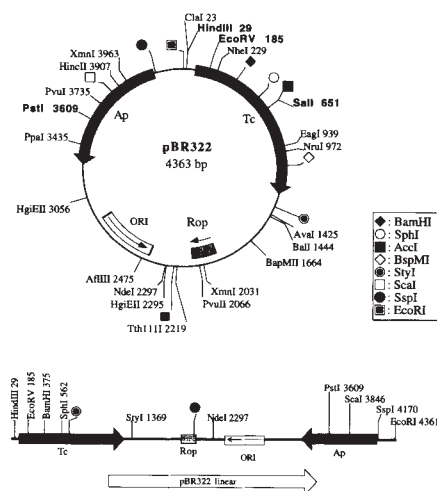


Brown-Out all-purpose stain remover.

a soap or surfactant but a specially formulated agent that chemically removes developer and fixer stains. The solution is said to be effective on both new and older stains that have 'set' in clothing and on equipment and surfaces in the laboratory.

■ Software

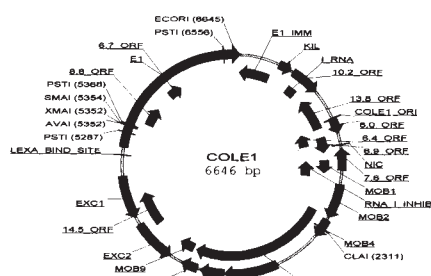
MacPlasmid version 2.0 from CGC Scientific is the latest version of a Macintosh-based **plasmid map drawing program** for both circular and linear plasmid maps (*Reader Service No. 102*). Tools are provided for the entry and accurate placement of restriction sites and genes on a map (which can be drawn with various styles, colours, patterns and symbols). Circular maps can be rotated and converted to linear maps at any point in the map. The ability to cut/copy/paste map features within or between maps and to delete/insert/invert map regions allows new maps to be constructed from pre-



pBR322 drawn using MacPlasmid. The linear map is shown below.

existing ones. MacPlasmid provides functions to import sites from PICT files generated from other DNA analysis programs (such as DNA Strider) and to read genes and other features from feature tables of GenBank and EMBL sequence files. Maps can be exported to other programs via the PICT format and printed for use in publications or for presentation purposes. A demonstration disk is available on request.

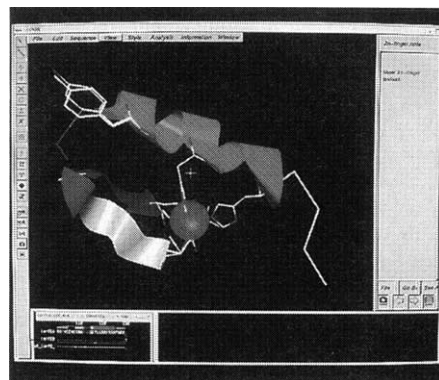
New from InforMax is **Vector NTI molecular biology software** (*Reader Service No. 103*). This program is available in a Windows and a Macintosh version. Vector NTI's main function is to design new genetic molecules according to the user's specifications. It recommends necessary cloning steps. Users can specify what methods are to be used by Vector NTI for fragment isolation, modifications to the termini and ligation of the fragments. The system has incorporated about 3,000 rules for genetic engineering. The database (which features 80 of the most commonly used vectors) allows molecules to be imported from Gen-



Vector NTI from InforMax is now shipping for Windows and the Mac.

Bank (GCG), EMBL and ASCII files. All molecules can be analysed to identify sequences for PCR primers, hybridization probes, sequencing primers, restriction sites, open reading frames and sequence motifs. Version 2.0 costs US\$995 (IBM PC); \$1,495 for the Mac version.

Molecular Applications Group is now shipping **LOOK — molecular modelling software** developed specifically with the molecular biologist in mind (*Reader Service No. 104*). Designed to help molecular biologists use protein modelling to design experiments and interpret their results, LOOK provides the tools needed to focus an experiment, to test hypotheses and to extend and

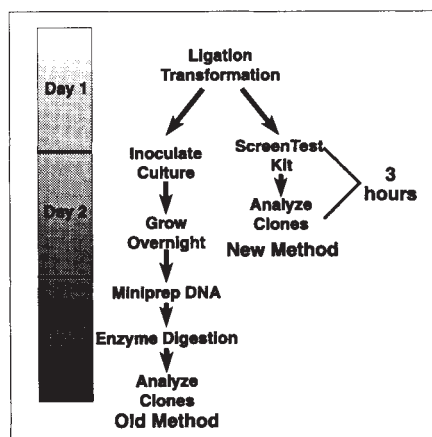


Mutant structure of a zinc-finger in the yeast transcription factor ADR1. The superimposed tyrosines at position 118 are the mutant sidechain orientation calculated by LOOK and the solved structure of the same mutant H118Y.

stimulate research hypotheses. Site-directed mutants can be modelled in minutes, says MAG. An accessory package for homology modelling is available for modelling a protein of unknown structure to an existing X ray or NMR structure. In addition, LOOK allows the user to click on a structure and find out what is already known and published from a custom-tailored literature database. The program runs on all DEC, Alpha or SGI workstations. It can also run on the Mac or IBM/compatible PC using a client-server setup with a workstation.

■ Kits

The **ScreenTest recombinant screening kit** from Stratagene provides a method for three-hour, PCR-based recombinant insert analysis directly from transformed colonies (*Reader Service No. 105*). Using this approach, the need for overnight inoculations, DNA miniprepations or restriction enzyme digestions has been eliminated. The kit's



The number of steps involved with the ScreenTest method versus DNA miniprep analysis and restriction enzyme digestion.

primers contain highly conserved complementary sequences and were designed to be asymmetrical to the multiple cloning sites in plasmids, making it possible to determine the presence and orientation of an insert by agarose gel analysis of the PCR-generated products. When the ScreenTest primer set is combined with a user-defined insert-specific primer, characteristic banding patterns will verify orientation of the insert. The kit can be used to screen for recombinant clones from most ColE1 plasmids or excised Lambda ZAP insertion vectors. Stratagene has also launched the Chameleon double-stranded, site-directed mutagenesis kit which allows single- and multiple-base substitution, insertion and deletion mutations in most double-stranded plasmids, eliminating the need for laborious subcloning steps. The kit's enhanced site-elimination mutagenesis procedure is designed to overcome the limitations of other methods by introducing a new *mutS* host strain, *XLmutS*, which is *EndA*⁻. The *endA* mutation removes an endonuclease that degrades miniprep DNA, improving the yield and quality of mutated plasmid DNA. Transformation of mutated plasmid DNA into the Chameleon kit's competent *XLmutS* cells, followed by transformation into *Escherichia coli* such as XL-1-Blue, is said to result in mutagenesis efficiencies of 70–90 per cent. Mutations ranging from a single base change to deletions as large as 48 bp have been generated.

Perkin-Elmer has introduced two new protein analysis kits for capillary electrophoresis intended for use with its ABI 270A-HS CE System (Reader Service No. 106). The MicroCoat Protein Analysis Kit provides a means of analysing a broad range of proteins over a wide pH range. This kit is designed to offer minimal pre-run preparation, direct detection and non-destructive sample analysis. It includes the MicroCoat Charge Reversal Reagent, capillaries, a neutral marker, standards and a protocol. The ProSort SDS-Protein Analysis Kit is intended for quantitative molecular weight-based

separation. It can be used to run at least 400 analyses of proteins ranging from 14 to 205 kilodaltons and glycoproteins. To complete its protein analysis and characterization system Perkin-Elmer also offers the ProFocus Isoelectric Focusing Kit for determining the isoelectric point of proteins and peptides over a wide linear pI range.

Kretech Diagnostics has developed the Bio-ULS biotin labelling kit which is based on the company's Universal Linkage System (Reader Service No. 107). The system uses a special platinum compound that has two free binding sites, one of which is used to bind a marker group. Bio-ULS is an example of such a platinum/marker complex. The other free binding site is used to bind the complex to DNA, where it binds to guanine groups. With this procedure, Bio-ULS is added to DNA and incubated for 30 min at 85 °C. After incubation, the labelling reaction is stopped by adding a blocking solution, after which the labelled product can be purified. The method is suitable for the labelling of primers prior to amplification. The Bio-ULS-labelled probe can be used in DNA *in situ* hybridization in tissue sections/smears, RNA *in situ* hybridization, chromosome detection and filter hybridization.

GenHunter's RNAimage kit is a complete system for differential display of mRNAs (Reader Service No. 108). It is based on a third-generation of differential display technology featuring the one-base anchored oligo-dT primers. As such, it is designed to overcome the redundancy and under-representation in gene detection experienced with the two-base anchored primers. The one-base anchored primers are said to be highly selective in subdividing the mRNA species into three groups each with G, T or C located immediately upstream of their poly(A) tails. This improvement is designed further to decrease the number of reverse transcription reactions needed for each RNA sample without any primer degeneracy. The introduction of a restriction site at the 5' ends of both anchored and rationally designed arbitrary 13mers ensures increased reproducibility of differential display patterns, allowing differentially expressed genes to be more readily identified and manipulated. Also available from the company, a new PCR-TRAP cloning system. The

system uses a third-generation of cloning vector that features positive selection for direct cloning of PCR products. Only vector inserts through blunt-end ligation confers antibiotic resistance so any colony that grows on a tetracycline plate should contain plasmid with DNA insert.

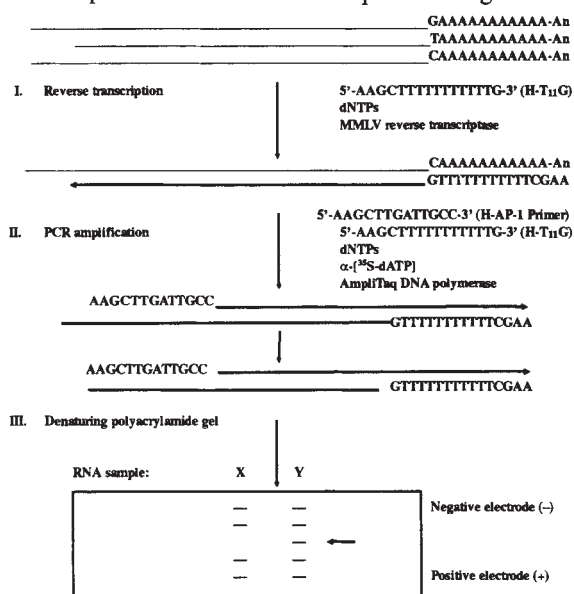
Buffers

Omnibuff is Wak-Chemie's new universal wash buffer for use in *in situ* hybridization for both chromosome analysis and tissue sections (Reader Service No. 109). It is designed to reduce the number of washing steps and the time required for stringency washing in post-hybridization, to eliminate washing steps with formamide and to be used with detection systems that are based on fluorescence or enzymatic visualization.

Cambio is marketing the PARR (Polymerase Assisted Repair and Replication) buffer designed to improve the yield of PCR products (Reader Service No. 110). There are a number of active ingredients, including reagents that enhance the ability of the polymerase to bind substrate. PARR buffer is available with or without magnesium in 1-, 10- or 100-ml packs — orders of 1 litre or more can be custom-made.

Purification

CrystalClear Kits from Molecular Bio Chemical have been designed to purify and concentrate double- and single-stranded DNA or RNA (Reader Service No. 111). Direct purification from all types of electrophoresis gels, cleared cell or tissue lysates and solutions is achieved with stated recoveries in the 70–90 per cent range.



Schematic representation of differential display using GenHunter's RNAimage kits. Three, one-based anchored oligo-dT primers with *HindIII* site are used in combination with a series of arbitrary 13mers containing *HindIII* site to reverse transcribe and amplify the mRNAs from a cell. Reamplified cDNA probes cloned into PCR-TRAP cloning system can be cut out with *HindIII* restriction enzyme.

The Easy-DNA kit from Invitrogen provides a **method for isolating genomic DNA** (*Reader Service No. 112*). The kit is designed for use with blood, cells or tissues, using small (1–100 µl of blood) or large (0.5–1 g of tissue) samples. The four-step procedure — lyse, precipitate proteins, extract and precipitate the DNA — is said to take 90 mins and yield genomic DNA between 100–200 kb. Invitrogen says that the genomic DNA isolated using this method produces discrete PCR products and also DNA hybridization products on Southern blots. Typical yields are said to be 25 µg for 1 ml of blood and 150 µg for 50 mg of liver tissue.

Polysciences offers high purity **guanidine hydrochloride and guanidine thiocyanate** bioreagents, with stated purities of greater than 99 per cent and 98 per cent, respectively (*Reader Service No. 113*). These products are useful in RNA isolation procedures. They have been tested for DNase, RNase and protease activity and are available in small research quantities as well as in bulk. The company can meet requirements for custom synthesis and packaging of these ultrapure bioreagents.

The Scientific Imaging Systems group of Eastman Kodak introduces its new IBI Yeast N-Terminal FLAG expression kit for immunological **detection and affinity purification of recombinant proteins** (*Reader Service No. 114*). The system is based on the fusion of a low molecular weight, hydrophilic, eight amino acid FLAG peptide to the amino-terminus of a cloned protein expressed by the YEpFLAG-1 expression vector. The secreted FLAG fusion protein may be affinity purified in a single step using the anti-FLAG M1 monoclonal antibody coupled to an agarose support. Following elution under mild conditions, a purified native protein is recovered by removing the FLAG peptide with enterokinase. Anti-FLAG monoclonal antibodies may be used for specific detection of the FLAG fusion protein in commonly used immunological procedures. The system includes no induction and provides a means of protein processing similar to animal cells.

RNaseZAP from Ambion is a new **formulation for RNase decontamination** designed to remove even trace amounts of ribonuclease from glassware, plasticware and equipment (*Reader Service No. 115*). Even trace amounts are known to lead to lower yields in *in vitro* transcription reactions, degradation of RNA during purification protocols and variable results in ribonuclease protection assays and northern blots. The solution, which contains three active ingredients that are said to destroy RNase on contact, is supplied in a 250 ml bottle and is applied directly to the surface to be cleaned. There is no incubation time once the surface has been covered with

RNaseZAP; it simply has to be rinsed twice with distilled water.

■ Equipment

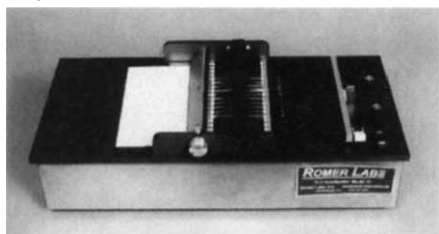
Owl Scientific announces the new Lightning Volt OSP-250L **power supply** (*Reader Service No. 116*). This multipurpose power supply should be useful for DNA and RNA submarine gels, mini- and large-format vertical systems and electroblotting with small semi-dry or tank blotters. The OSP-250L can operate in both constant voltage and constant current modes with automatic crossover. The maximum settings are 250 V, 500 mA and 125 W. The 24-h timer counts up or down and an alarm sounds and the voltage shuts off when the clock reaches zero. The system restarts automatically if



Lightning Volt OSP-250L multipurpose electrophoresis power supply.

power is temporarily interrupted. Run settings are retained even if the power supply is switched off. Four output connectors allow multiple chambers to be run simultaneously. Safety features include a sensing circuit that shuts off the voltage if the chamber lid is removed or if the circuit is broken.

Analtech has produced the Autospotter — a semiautomated **applicator for thin layer chromatography plates** (*Reader Service No. 117*). Autospotter uses TLC syringes with blunt Teflon-tipped needles. It



Analtech's TLC autospotter.

can apply up to 18 samples at a time and at variable rates. An integral heater strip runs beneath the TLC plate at the point of sample delivery to aid in solvent evaporation. Delivery rate and temperature of the heater strip can be adjusted to vary the size of the sample zone.

Isco's Foxy Jr **fraction collector** now has the ability to hold two 96-well microplates, allowing collection down to a single drop for small-scale applications such as micro-chromatography and automating



Isco's Foxy Jr fraction collector now handles microplates and has documentation capabilities.

ELISA assays (*Reader Service No. 118*). Another new feature is the ability to document, via an external printer, system automation methods as programmed on the Foxy Jr keypad. Data typically includes timing of valves and pumps for gradient formation, buffer change and sample addition, as well as fraction collection procedure and other events.

Amersham has refined the Hypercassettes line of **autoradiogram cassettes** with colour choices and wipe-clean labels (*Reader Service No. 119*). The wipe-clean labels allow identification and experimental details to be recorded with a permanent marker. The colour choices of red, blue or neutral colours are designed to help users separate out batches of work to ensure correct exposure times; they also provide a means of readily differentiating between radioactive and non-radioactive applications. These cassettes still come with aluminium frames with chip-proof surfaces and stainless steel hinges to ensure tight closure. This design is said to maximize contact between film and sample.

New from Advanced Biotechnologies — thin-walled **micro-tube plates for primer extension procedures** (*Reader Service No. 120*). These plates have a 12-strip, 96-well configuration (0.2 ml tube volume) and are supplied pre-assembled in a patented injection molded strip retainer. The plates are said to fit both V-bottomed and round-bottomed, thermal cyclers with a block configuration. They can be sealed with the company's strips of eight polypropylene caps, heat-sealing film, tape or the range of sealing mats that are on the market. Micro-tube plates are suited for use with silicone/rubber mats and feature a raised tube rim for enhanced contact with the mat. They can be autoclaved and are available in sterile or non-sterile versions. To help in sample identification, the plates are grid-referenced and colour-coded.

■ Fluorescence

Two tungsten-halogen-xenon lamps and non-fibre optics are design features of Dynatech's new Fluorolite 1000 **dual-lamp fluorescence reader** (*Reader Service No. 121*). With this device, the company says that

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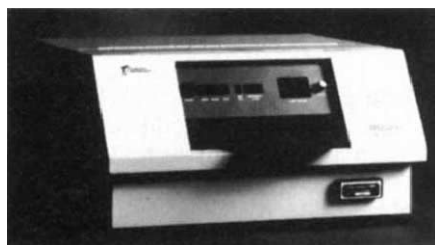
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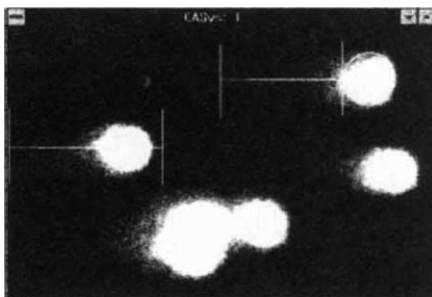
Reader Service No.426



Dynatech's dual-lamp fluorescence reader.

10 pg ml⁻¹ Rhodamine 123, 5 pg ml⁻¹ Hoechst 33258 or 250 pg ml⁻¹ Neutral Red can be detected in culture. Stated applications for the Fluorolite 1000 include cytotoxicity assays involving cell membrane, cell viability and mitochondrial activity studies, PCR product analysis and DNA hybridization, monitoring bacterial growth and lymphocyte differentiation using fluorescent monoclonal antibodies. The device has an extended working range of 300–900 nm. User control of lamp voltage is designed to allow optimum gain adjustment over the system's entire dynamic range. The unit's stated resolution over the range – 500 to 4,094 fluorescence units is linear to ± 3 per cent and repeatable to ± 5 per cent. Dynatech says that the Fluorolite 1000 can accommodate any 96-well microplate. But, for low-level background and reproducible readings the company recommends its MicroFluor system. In addition, 6-, 12-, 24- and 48-well plates and 35-mm Petri dishes can also be read, as can filter-bottom plates or plates with lids, magnetic particles or micro-beads.

Synoptics has released an upgraded version of the CASys Comet Measurement System for the **comet fluorescence assay technique** (Reader Service No. 122). This Windows-based system includes a means of measuring comet area, comet length (true longest dimension), leading edge moment, centre of head movement, comet width and



Upgraded comet measurement system.

total DNA, and, now for the first time, comet tail length and tail moment. Comet tail length is measured with respect to the centre of the comet head. The tail moment gives a measure of the amount of movement of DNA from the head end of the tail to the opposite end. The system features a high-sensitivity, low-light integrating CCD camera. A variable exposure facility allows an integrated image to be produced on the surface of the camera sensor before transfer to the image store.

The RF-Mini 150 from Shimadzu provides **fluorometric quantitation of DNA, RNA or fluorescent tags** (Reader Service No. 123). Shimadzu says that high sensitivity measurements from microgram to picogram levels are attained through routine analyses of DNA using Hoechst dye and RNA/DNA with ethidium bromide. Standard features include gain settings, a timer and an internal clock for automatic time and date stamps. Data output with the RF-Mini 150 is accommodated by the built-in RS-232C or analog output to external printing devices. Excitation and emission wavelengths can be selected by interchangeable filters, available in application packages to cut down on the guesswork.

Separation

Pierce's XTreme **spin column kit** is designed to remove low molecular weight contaminants from DNA solutions or gels (Reader Service No. 124). The kit can be used to remove dNTPs, primers and salts from nucleic acids that are 100 bp to 20 kbp in length. Quantities ranging from 1 ng to greater than 10 µg can be recovered and with stated yields in excess of 90 per cent. Suggested applications include cleaning up plasmid miniprep DNA, purification of PCR products from primers and dNTPs, removal of unincorporated dNTPs from DNA labelling reactions, desalting DNA solutions in preparation for subsequent enzymatic reactions and purification of DNA fragments from high-resolution agarose gels.

Merck has introduced a stable 300 Å **wide-pore silica gel** with a RP18 stationary phase intended for the analysis of peptides and t-RNA fragments up to a molecular weight of 20,000 (Reader Service No. 125). The specially treated silica is designed to provide better peak shape and separation, particularly of basic peptide oligomers. Scale-up is possible.

PhosphoMagnaCel is Scigen's new derivatized **ion exchanger for the purification of DNA-binding proteins** from cellular extracts using magnetic separation and a single-tube format (Reader Service No. 126). It consists of negatively charged phosphate groups attached to sugar residues on a paramagnetic support. Binding capacity is said to vary, depending on the salt concentration and pH, with a stated maximum capacity of about 100 µg of protein per milligram of PhosphoMagnaCel. Many basic proteins such as lysozyme and nuclear proteins can be purified or enriched from crude mixtures using this approach. It may also be useful in the purification of recombinant DNA-binding proteins from *Escherichia coli*. □

These notes are compiled by Diane Gershon and Brendan Horton with information provided by the manufacturers. For more details on any of the products featured, fill in the reader service card bound inside the journal.